LECTURES ON THE
SCIENTIFIC BASIS OF MEDICINE
1954–55

British Postgraduate Medical Federation
University of London

LECTURES ON THE SCIENTIFIC BASIS OF MEDICINE

Volume IV

1954–55

UNIVERSITY OF LONDON
THE ATHLONE PRESS
1956

British Postgraduate Medical Federation
University of London

LECTURES ON THE SCIENTIFIC BASIS OF MEDICINE

Volume IV

1954–55

UNIVERSITY OF LONDON
THE ATHLONE PRESS
1956

Published by
THE ATHLONE PRESS
UNIVERSITY OF LONDO
at 2 Gower Street, London, W.C
Distributed by Constable & Co.
12 Orange Street, London, W.C.
U.S.A.
John de Graff Inc.
31 East 10th Street,
New York, 3

Printed in Great Britain by
WESTERN PRINTING SERVICES LTD
BRISTOL

PREFACE

THE prefaces to earlier volumes of *Lectures on the Scientific Basis of Medicine* have described in detail the scope and purpose of the lecture courses arranged each winter by the British Postgraduate Medical Federation for young research workers, and future consultants and specialists. The publication of an annual volume made up of a selection of lectures from each course provides the opportunity to reach a wider audience than that for which the series is primarily though, of course, not exclusively designed. The published volume also helps to assist continuous familiarity with the series as a whole, and this is particularly to be desired inasmuch as each individual course is planned, not in isolation from earlier and later courses, but as a stage in a progressive survey of developments concerning the fundamental principles of medical science that may be applicable to clinical practice.

In introducing this fourth annual volume it is therefore pertinent to remark that since the commencement of the series a number of methods of investigation that were the subjects of lectures have become the common tools not only of laboratory research but also of clinical investigation; such are chromatography and the use of radio-isotopes, the latter being freely used also in the treatment of disease. The study of macromolecules of biological importance by means of x-rays and electron-microscopy which was discussed in an early lecture in the series now provides a basis for the more intensive study of disease processes, and is illustrative of the growing need for the application of physical methods to the elucidation of biological processes. Physiology and biochemistry continue to contribute substantially to the clinician's understanding of the problems met at the bedside and to the methods by which he investigates his patients and arrives at a clearer understanding of health

and disease; the lectures in these fields show that for such a purpose knowledge of the functions of organs no longer suffices, but that the cell, its structures and their functions, are to an increasing extent becoming the basis of medical science.

The contents of Volume IV illustrate these trends, from A. V. Hill's opening address entitled 'Why Biophysics?' to the final lecture by A. V. E. Pearse on 'Histochemistry and its Application to the Basic Sciences'. The molecular structures of cyto-proteins, nucleo-proteins and plasma-proteins are the subjects of other lectures that show how the physiologist now deals with the infinitely small. Trauma is the simplest form of disease and a number of lectures deal with the reactions of cells and biochemical substances to injury. The study of the cell is further exemplified by the lecture by C. H. Andrewes on '*The New Look in Virus Research*', and the progress towards a solution of the cancer problem is described this year by F. Bergel in his lecture on 'Some Chemical Aspects of Abnormal Growth'.

Advances on similar lines are taking place in many other fields of research of importance to clinical science. A number of these are represented in the 1955–56 series of lectures and will appear in the fifth volume now being prepared for publication, which it is hoped will be ready early in 1957.

FRANCIS R. FRASER
Director, British Postgraduate Medical Federation

13 March 1956

CONTENTS

NOTE

The lectures printed in this volume were delivered on the following dates:

I.	19 October 1954	XI.	9 November 1954
II.	1 March 1955	XII.	25 January 1955
III.	15 February 1955	XIII.	11 November 1954
IV.	7 December 1954	XIV.	17 February 1955
V.	10 February 1955	XV.	20 January 1955
VI.	4 November 1954	XVI.	18 January 1955
VII.	24 February 1955	XVII.	30 November 1954
VIII.	8 February 1955	XVIII.	28 October 1954
IX.	21 October 1954	XIX.	22 February 1955
X.	16 November 1954	XX.	23 November 1954
		XXI.	18 November 1954

I

Why Biophysics?

A. V. HILL

DURING the next five months the British Postgraduate Medical Federation has arranged thirty lectures on 'The Scientific Basis of Medicine'. The presence of the Vice-Chancellor today, and the fact that this address is described as an Inaugural Lecture, might suggest that it is, in some special way, to be an introduction to the remaining twenty-nine. Only in the broadest sense can that be true, only if the ideas which are to be laid out are taken as having a general application (when necessary, with a suitable change of phrase) to any scientific discipline from which medicine can derive advantage. The title chosen, *Why Biophysics?*, suggests an inquiry into what role a particular branch of science, one which only recently acquired a name and personality, can play in medical progress and practice. Looking through the titles of this series in the last five years one finds that, whereas a large proportion are obviously derived from physiology or biochemistry, very few could reasonably be called biophysics—even if one includes a lecture by the President of the Royal College of Physicians on 'Mind and Matter'! This is natural and proper, for physics hitherto has not had the same intimate connexion with medicine that physiology and chemistry have had: its applications have rather been to engineering, while its ideas have been derived not at all from biology but from mathematics, astronomy and philosophy It is true that physical ideas and physical instruments and methods are fundamental to nearly all fields of science: but it is only in recent times that separate branches of physics have been recognized in which the primary object was

not physics itself but some other subject—for example, astrophysics aiming at the problems of astronomy, geophysics at those of the earth, physical chemistry at those of chemistry and now biophysics at those of biology. Chemistry also has proliferated—into geochemistry, biochemistry, and a host of practical applications, the object of which is not the solving of purely chemical problems but the use of chemical ideas and methods for understanding, or doing, something else.

Biophysics is a newcomer; so was biochemistry forty to fifty years ago. This does not mean that chemistry was not applied to biology and medicine long before, in fact chemistry and medicine grew up together: but it does mean that biochemistry began clearly to emerge as a separate and independent discipline, with its own ideas and methods, early in this century. To this audience it is unnecessary to insist that biochemistry is *not just chemistry; to take the biological idea out of it would be* like depriving a man, not of his clothes but of his skin—he might be an interesting object of study but he would cease to be a man. In the same way biophysics is not just physics, but a *sturdy and promising child of physics and biology which has* set out on the same road of independence as biochemistry did forty or fifty years ago.

The idea of function, of organization, of design, is an essential part of biology as it is of engineering. In physics and chemistry, apart from their applications which are really engineering, it is meaningless. Whatever his philosophical or theological views, it is sensible and expedient for a physiologist, using that term in the wider sense, when investigating an organ, a structure, a response or an adaptation, to ask what its functional significance is, its relation to other parts of the machinery, its purpose in connexion with behaviour, survival or inheritance. If his conscience, or his politics, forbids that much teleology, he had better take up something else, for in biology he will miss most of what is interesting. The biochemist is not merely a chemist working at the chemistry of material that was once alive, or was produced by living activity: he is primarily concerned with processes that go on within the living organism, and their dynamic connexions with function or behaviour. The comparative study

of animals from the functional standpoint turns up innumerable examples of what any engineer, faced with them, would assume to be highly competent design—and botanists surely would insist that the same is true of plants. After all, evolution has been at work for a very long time, and we cannot neglect its consequences.

WHAT IS BIOPHYSICS?

The term 'biophysics' is coming today into common use, but as yet no clear definition of it has emerged. The emphasis must clearly be on the 'bio', on function and structure viewed through physical spectacles and investigated by physical ideas and methods. The word 'definition' implies setting limits, and it is convenient to start defining biophysics by reciting many things which it is not. One thing it clearly is not is a second-rate branch of physics, a haven of refuge for indifferent physicists. It does not consist of teaching physics to medical students. It is just as unsuited—and some may think this a hard saying—to people who know no biology as to those who know no physics. It does not consist of constructing, or maintaining, physical equipment for use by anatomists, biochemists, physiologists or clinicians. The employment of physical instruments in a biological laboratory does not make one a biophysicist—otherwise any user of a microscope, a balance, an X-ray equipment, a Geiger counter, or a pH meter, would drop automatically into that class. The crystallography of material of biological origin is not in itself biophysics, any more than organic chemistry is biochemistry. Using amplifying valves or radio-isotopes, or working on muscles and nerves, does not confer any biophysical status It all depends on the motive, on the idea, on the method and manner of approach.

Science does not operate only in separate compartments of knowledge, and many of the best discoveries emerge from the borderland between several of them One reason, however, for adopting a new name, or inventing a new organization, may be to find room for people whose special knowledge or talents cannot fit into the accepted scheme. The growth and recognition of biochemistry made it possible for people trained first in the discipline of chemistry to work in the biological field, on equal

terms with those coming from medicine or biology. Few of these would have found a home and opportunity in laboratories of physiology, anatomy, pharmacology or pathology, where most appointments were by tradition, and still are, reserved for medical graduates. Few of them could have established themselves in laboratories of zoology or botany, the scale and scope of which were usually too small. In the end it was better so, for biochemistry has emerged as an adult subject, asking nobody's leave to work at any of the problems of biology by chemical methods and with chemical ideas. Today, forty to fifty years later, the same course is being followed by biophysics.

For some time now, and particularly since the recent war, an important number of young physicists have got hold of the notion that some of the most fruitful outlets for physical ideas and methods are likely to be found in biology. Perhaps this is partly due to a reaction against the aggressive dominance of nuclear physics, and a reluctance to become a cog in that particular machine: partly I am sure it has been that biophysics offered scope to adventurous and adaptable minds. Another reason, probably, was the resource and initiative with which many young biologists mixed up with physicists, in telecommunications and in operational research and the like, during the war. In biology one has to live by one's wits, competing as in a game with living material which is always to some degree unpredictable: one does not have to go humbly to a theoretical biologist to plan the strategy of one's research. This tends to produce a quick response to the unexpected—which is useful in war, and very often in research. Contact with such people may have given some young physicists an impression that biology, after all, can be quite a respectable subject, with many exciting problems waiting to be tackled by new and adventurous methods. It has even come about that eminent professional physicists are ready today to admit the interest and difficulty of biological problems and the intellectual and experimental skill required for tackling them. That was not always so, and again the analogy with biochemistry is apt. At one time the professional chemist was liable to regard biochemistry as a nasty messy subject, not worthy of his distinguished attention.

Today many of the leaders of chemistry are working in fields that verge on biochemistry—indeed to such an extent that some of them would like to appropriate the subject as their own, and biochemists have had obstinately to maintain their identity against the acquisitive dominance of chemistry. Out of the frying pan into the fire might have been the fate of biochemistry when it got away from physiology. For many years physicists and mathematicians have been among my closest friends, and one of my functions has been to keep them humble by reminding them now and then that other subjects than theirs are intellectually quite as respectable, experimentally much more difficult, and generally far more amusing. The simplified problems of physics require only a small part of one's thinking apparatus, used no doubt very intensively: those of biology demand a much greater share of one's resources. Today my friends are more respectful; naturally they do not like their best pupils leaving them to go off into biophysics, but at least they do not regard such defection as an irretrievable disaster to science!

It would be a damaging mistake, however, to suppose that physics, in the narrower sense, is the only partner of biology in the new field of biophysics Most vital processes take place in an aqueous medium, in which chemists are much more at home than physicists. There is no sharp boundary between chemistry and physics, nor should there be between biochemistry and biophysics. The natural division, in general, would be for biological problems involving physical chemistry to be drawn towards biophysics, as those involving organic chemistry would go to biochemistry. The physical chemists who turn over to biophysics may be expected to make at least as great a contribution as the physicists proper. With nuclear physics, as such, which tends at present to dominate physics, biology is little concerned Biological processes are based on molecular change, and only to the extent that the chemistry of molecules is determined or affected by the physics of their atoms does the latter impinge directly on the interests of biologists. Indirectly, of course, by the tools it provides and the effects of its products, nuclear physics is of the greatest concern to biologists—as it is to everybody· but the fact remains that vital processes

themselves, to an overwhelming extent, are more of a chemical, or a physico-chemical, than of a [illegible]
deed [illegible]
I [illegible] little reason for a separate existence.

Twenty-four years ago, almost to a day, I addressed a gathering in Philadelphia, at the opening of a new laboratory of Medical Physics of which my friend D. W. Bronk was head. Much good work, largely of a new scientific flavour, has come from that laboratory. Twenty years later, again almost to a day, I took part in the opening at Johns Hopkins University of another new laboratory of biophysics, also organised by Dr. Bronk. In the United States today there are many departments of biophysics, not always under that title, in which physical ideas and methods are being applied to biological problems. The great developments of nuclear physics in America, as here to a somewhat smaller extent, have led to a large expansion of what can be called radiobiology: this is aimed partly at the protection of the human body from the effects of radiation, partly at improvement of medical treatment but [illegible]
at solving the fundam[illegible]
of various kinds [illegible]
ance. This, how[illegible] aspect of biophysics: another important one is the study of the minute structure of living cells, which is being pursued in America for example at F. O. Schmitt's laboratory at the M.I.T., and here in London in Randall's laboratory at King's College. Three years ago the first regular university department of bioph[illegible]
was start[illegible]

[illegible] groups
[illegible] laboratories and centres, here and in other countries, under many titles. It is not at all necessary that there should be departments of biophysics in every univer[illegible]
any more than there [illegible]
thing is that a f[illegible]
act as nuclei for [illegible] research, and to give the subject a place on the map, a personality and status which will draw

young people of the right kind into its pursuit. The process has started well, and can be left to develop largely of itself, with sympathetic help but without too much overhead planning. One need not advocate, for example, that there should be an International Union, or International Congresses, of Biophysics: it can safely find its scope, at least for a long time yet, within those of Physiology, Biology and Biochemistry.

With all these qualifications about what biophysics is not, may I try to define what it is: as the study of biological function, organization and structure by physical and physico-chemical ideas and methods. There is nothing very interesting or original about that; except perhaps the fact that ideas are put first; for physical methods and instruments of every kind may be used in any field of research. Biological phenomena, like many others, show aspects and relations susceptible of physical analysis and interpretation. It is by the choice of problems and by the intellectual processes with which they are formulated and attacked, more than by the particular techniques employed, that a subject can be most clearly defined. There are people to whom physical intuitions came naturally, who can state a problem in physical terms, who can recognize physical relations when they turn up, who can express results in physical terms. These intellectual qualities, more than any special facility with physical instruments and methods, are essential to the make-up of a biophysicist. Equally essential, however, are the corresponding qualities, intuitions and experience of the biologist. A physicist who cannot develop the biological approach, who has no curiosity about vital processes and functions, who is not willing to spend time in learning the habits of living things, who regards biology simply as a branch of physics, has no important future in biophysics.

In speaking of the intellectual side of the physical approach to biology you must not suppose that I underrate the technical side or imagine that theoretical physics has more than an occasional role at present to play in biology. So far, indeed, from underestimating fine physical techniques applied with the skill and understanding that come from experience in handling living material, one would insist rather that progress is waiting

on their application, and that biological literature is beset by the results of imperfect experimentation. I was urging only that the primary condition is the right intellectual approach. Granted that condition, achievement can come only by highly skilled and often laborious experiments, laborious because of the essential lability of the material. In such experiments the instruments and methods must often be adapted to the object investigated, so that the principles of their design and working must be understood. When one is dealing with units of extremely small size like living cells, personal skill becomes of primary importance. It cannot usually be replaced by statistical methods, important as these are in their proper place. The chief concern in the development of biophysics is that those skills should be acquired by people who start with the right intellectual approach, physical and biological.

Let us think for a moment how the process of investigation goes. In the function or structure to be studied, some factor is chosen which not only is open to physical description, or attack by physical method, but also if so treated may lead to unambiguous results. It is all too easy—and frequent—to make beautiful experiments which cannot, in any case, tell us anything: or perhaps may tell us about something in which we are not interested. I recall experiments which purported to measure the elasticity of muscle, but gave in fact, rather inadequately, the constant of gravitation: and others which were intended to verify a particular theory of colloid behaviour, and really proved nothing except the second law of thermodynamics It is all too easy to employ fashionable physical devices for purposes better achieved by simpler traditional methods. For example, some years ago, an old-fashioned gas regulator with a tapered jet was found to beat all the best electronic devices of its day for maintaining a constant temperature in a water bath. The use of the latest physical methods and devices does not make one a physicist, and the employment of such things in a biological laboratory is not necessarily biophysics. The man who has physical ideas, who can see physical problems, who recognizes the opportunity of physical investigation when it turns up, who understands and can use physical techniques, such a man

can find unlimited opportunity in biology, if—and it is a fundamental IF—if he is willing to learn something also of the facts and philosophy of biology, to be apprenticed for a time in a biological workshop. Some of the most accomplished contributors to what is really biophysics, though it may be practised in laboratories under other names, started in fact as biologists: and a complete department of biophysics really requires both kinds—with a reasonable admixture of engineers!

Relation of Biophysics to Biochemistry

In one important respect the roles of biophysics and biochemistry are complementary. The single unit in biology, the living cell, is very small: the quantities involved in its processes are beyond the range of ordinary chemical measurement. It is necessary therefore to use large numbers of cells, similar as far as possible, and to accept a statistical blurring of the result. For biophysical purposes, however, it is frequently possible to use single cells and to examine the individual process. with the limitation always that most physical methods are chemically non-specific, so that interpretation in chemical terms is bound to be indirect. In biochemistry, even accepting the necessity of working with a large number of cells, there are two further limitations; its methods are usually insensitive and very slow, and many of them requre the destruction of the tissue for their application. The astonishing thing is that biochemistry has, in fact, been able to achieve so much. In comparison, the methods of biophysics may be very sensitive and rapid, and often leave the tissue unaltered so that observation can be continued: but they are not applicable to all problems and—one must insist—they are nearly always chemically unspecific.

Let us illustrate the contrast between biochemistry and biophysics by the example of muscle. Here the fundamental unit of response is the single muscle twitch, a very rapid affair involving only a minute chemical change. Physical methods are available of high sensitivity and speed which can give a quantitative picture, practically simultaneous with the events themselves, of electrical, optical, mechanical and thermal changes accompanying a twitch. Direct chemical methods, however, can tell

us nothing: in order to get measurable quantities it is necessary to subject the muscle to a sequence of stimuli spread over a longer interval. If it were possible to assume that a single chemical process was involved in muscular activity, that it went in one direction only and was not rapidly reversed, one might calculate that the chemical effect of forty twitches was simply forty times the effect of one twitch. In fact, however, the finer details of the complex sequence of chemical events are confused by repeating the stimulus many times; only the final or semi-final effects are accumulated. To take a simple example, the immediate physiological and biochemical consequences of running twenty metres at top speed would not be discovered at all by making a man run two kilometres as fast as possible and dividing the observed changes by 100. This difficulty of examining chemically the ultimate physiological events is widespread; in nerves, in muscles of all kinds, in the central nervous system, in all tissues in fact in which activity occurs in small discrete packets, rather than continuously. That still leaves available for biochemical study a variety of tissues in which activity is apparently continuous, but such tissues usually get into a steady state, in which the total metabolic effect over an interval is all that is measured, and the unravelling of intermediate processes has always been one of the greatest problems and has led to the greatest scientific achievements of biochemistry. In muscle, with its high rate of chemical turnover and the rapid changes and reversals involved in its unit of activity, our chemical knowledge has largely been derived from studies of isolated enzyme systems and chemical constituents. But that knowledge cannot be extrapolated backwards, without confirmatory evidence, to describe the actual chemical events of contraction. Such evidence can come only from methods of much higher speed and sensitivity than chemical technique is yet endowed with.

But—let us admit it humbly—to attempt to solve the problem by physical methods alone would be just as fantastic. Ultimately *the machinery itself is chemical in nature*, the fuel it uses for its recovery process is chemical, the 'acid' and the 'plates' of its 'accumulators' are chemical, the free energy of chemical

change provides the mechanical work, and various enzymes prescribe the course of the reactions. No physical methods conceivably available could give us the specific chemical information required to solve the problem properly. It is natural and healthy that biochemist and biophysicist should tease each other sometimes about the limitations of the other's methods: but each should be keenly aware of the limitations of his own and seek the co-operation which alone can solve their common problems. And perhaps it would be wise for them both to reflect, when they think they have solved them, that the biologist can still point out that although they may have found out how the machinery works—if indeed they have—that is very far from answering the question of how it grew and developed, how it maintains and adjusts itself, how its design is so singularly well-adapted to the needs and purposes of its owner.

The Philosophy of Biophysics

On that former occasion in Philadelphia in 1930 I spoke on the rather cryptic title 'The Physical Reasonableness of Life': it allowed me to expound a faith that no limit will be found at which the application of physical methods and ideas—and of course that implies chemical ones too—will be forced to stop in the investigation of living processes. I was at pains to emphasize that this certainly does not imply that biology will finally become simply physics and chemistry—at least as one knows those subjects now: indeed the boot is rather on the other leg, physics and chemistry have in the end a great deal to learn from biology, in their philosophy and ideas, as well as in their opportunities for research It is obvious indeed, at least to those biologists who know something about the properties of the nervous system, that physical theories and concepts can have no absolute validity apart from the brains that conceive and use them: if they *can* be conceived by the brain, it seems most unlikely that their pattern is not conditioned, and to some degree determined, by the properties and machinery of that organ. What we know of the working of brain and nervous system is largely due to the application of physical, particularly of electrical methods perhaps, in future, communication theory

will make its contribution, and that is a mathematical branch of engineering. But if we assume that a consistent theory of the natural world is ultimately possible, we have to admit that just as scientific instruments and engineering applicances are designed to fit the human senses and faculties that employ them, so scientific theories have to be made to fit the human brain that uses them. My physical friends are often rather indignant at any such idea: they have been brought up to believe that their postulates have some virtuous kind of absolute reality. The history of science scarcely bears out so naive an assumption; and when we know more about the mechanism of the brain I think we shall begin to see how it determines the pattern of any physical theory which it is humanly possible to conceive. This expectation accompanies an uncompromising conviction that the methods and ideas of the physical sciences are an unconditional necessity for biological progress: but it is part of an equally firm conviction that biology is not in the least danger of being swamped or subjected in the process. Physics and chemistry will dominate biology only by becoming biology. We can live in hope of the future unification of biological and physical science —but need not fear at all the dreadful prospect that life will be explained away in terms of present-day physics and chemistry.

At that meeting in Philadelphia twenty-four years ago the addresses were given in an auditorium outside which the police had placed a notice 'No parking here, place of amusement'. The compliment was highly appreciated. Nothing I could say today would meet the unwarranted expectation of the Philadelphia police any better than what I said then: in fact, one steadily becomes less amusing as one gets older—except perhaps unconsciously! This series of lectures is intended to deal with various aspects of science applied to medical progress, and since progress depends on available people it would be possible, and indeed in order, to make a number of platitudinous remarks about physics as a necessary ingredient in the training of a modern doctor. So far as these were true they would mostly be obvious, and if they were not obvious they would probably be false. The important thing to remember is that even medical students are human. In India in 1944, in reply to pressing

invitations from Indian friends which I should have loved to accept, I had often to insist that even a physiologist cannot be in more than two places at once. A similar limitation applies to medical students.

A few years ago I was temporarily involved with the problem of load carrying by the infantry soldier. In given circumstances, for a given individual there is an optimum load. With much less he will march and fight better—for a time—but soon he will have no food and water to march on, no weapons or tools to fight with: with much more load, he will march and fight worse, however well fed and armed. It is the same with the modern medical student: the poor boy, or girl, has a terrible load to bear anyhow and if you pile too much upon him (there is no danger of giving too little) you will make him unable to do the educational equivalent of fighting, namely to think, criticize and discuss. For that reason, much as we should like him to know more physics, chemistry and mathematics, more biology, physiology and anatomy, before we let him loose on his clinical course—much indeed as we should prefer him also to know more of literature, philosophy and sociology and to have a wider culture and experience—we must think of him, like the poor infantryman, as having an optimum load which it is improvident to exceed. That load, as with the soldier, has to be the same for all, so it must be within reason for the weaker individuals which means that the stronger ones could carry more. The extra physics, or the extra biology, or the extra culture and experience, must be regarded as an extra for those who can bear that particular burden easily enough. We must in fact provide opportunities outside the regular curricula for the more talented students to follow their natural bents. It would be the greatest misfortune if biophysics or biochemistry were to draw no recruits from medicine.

It needs no argument to show that physics and chemistry—or even mathematics in the special form of statistics—impinge every day on medicine and public health. It is essential that every doctor should have this much acquaintance with them that he knows *first* where to turn for help, whether to technician, specialist, manufacturer, or librarian, and *second*, and even more

important, not to be taken in by magic masquerading as science. In every field of medical science, and of medicine, we are witnessing the impact of chemistry and physics: on ways of looking at things, on research, on diagnosis and treatment. Indeed, the very nature of our modern society, based as it is on engineering applications of the physical sciences, is bringing a host of new problems, as well as of methods and equipment, to human biology and medicine. Indeed these applications of the physical sciences are providing some of the major problems which the world has now to face, problems which human biology and medicine can neglect only at their peril. The question is—what are we going to do about it, in connexion with medical education? The last thing one would want to do is to over-emphasize —as the public is apt to do—the place and importance of the physical sciences. The twenty-nine lectures, that will follow in this series, show how many subjects there are already to understand· and others might urge the importance of psychology, of social studies and of statistics, in the scientific basis of medicine. It is simply a question of how the load can best be distributed. We have to realize that the calling of the good physician requires every faculty of critical intelligence and knowledge, of sympathetic understanding, of skill and patience, that a human being can possess. In planning his education we must aim at a practicable optimum, not an impossible ideal· and that means a sensible compromise between all the things he has to learn and do, taking good care that he and she have time and energy enough left over to grow, as well as to be planned.

For some years, though a very long time ago, I was responsible for teaching physiology to medical students, and I frequently asked myself—and they asked me—what was the good of this or that? Most of them would have little direct use for many of these things in after-life. Might not time saved in such irrelevant studies allow them to acquire a little more personal skill in handling patients—which could serve them and the patients better. Such queries might seem particularly pertinent in respect of the physical sciences in the premedical curriculum. There are many answers to such a question, most of which are well known to you and for the most part to the students

themselves. It is unnecessary to an audience like this to emphasize that physics and chemistry are an essential background today to the biological sciences, that those are fundamental to medicine, or that physical and chemical methods of diagnosis and treatment make up a large part of modern medicine. One has rather to answer the objection that within the very limited load a medical student can bear there can be so little given him of each of these preliminary sciences, and that what he learns he soon forgets. We cannot expect him to be an expert in physics and chemistry, may not a little knowledge be a dangerous thing?

The answer I think is this, it depends on a property of the human mind, a property we all know, unconsciously perhaps, very well. Let me illustrate what I mean by personal experience. I learnt quite a lot of mathematics at school and in my first two years at Cambridge, but I have steadily been forgetting it ever since. One's mathematical knowledge seems to have a half-life of about three years! But it really hasn't mattered much. In countless jobs I have had to do, not only in physiology or biophysics, mathematical ideas and methods have been wanted. Somewhere deep in the brain the memory has continued of what mathematical ideas feel like, of what sort of problems can be tackled by mathematical means, of how to state a problem in mathematical terms, of where to look, either in books or to people, for a solution of it. My actual knowledge of mathematics for many years has been contemptible—yet I know how much the mathematical approach continues to influence the pattern of my work. Forgive me for referring to a personal experience; I do so because it is not really personal at all, but depends upon a general property of the mind. If a boy or girl has been brought up with the discipline of a mathematical, physical and chemical stiffening to his scientific training, he will find, whatever he does and however much he forgets, that a method of thinking and acting, of criticizing and assessing facts and theories, is available to him without which much of the world is meaningless: not only of the physical, but of the biological world, not only the world of our engineering environment, but that of medicine, of social relationships and of human behaviour.

That sounds like a tall order, but I think it is true. It implies that mathematics and the physical sciences can provide a framework for the other sciences that are to follow: and they should be learnt early. This means that scientific biology should come later; as I think it should, for scientific biology, as distinct from simple natural history, is more intelligible on a background of physical and chemical knowledge. Do not suppose that placing biology second in time means putting it second in importance. A certain amount of biology is an essential ingredient in education—even of a physicist, a politician, a parson or an engineer! But in the case of a professional biologist—as a doctor should be counted—it can be learnt best if the mind is prepared already by a reasonable preliminary dose of the physical sciences. It is hard to convince a medical student when he is twenty that a course in elementary mathematics is a useful preliminary to medicine: at that stage, indeed, it probably is not! It should have come six or seven years earlier. To some extent, and to a different time scale, the same is true of physics and chemistry. In a modern building the steel is no more important than the other constituents—but it is essential to put it in first.

Why Biophysics?

I have wandered from the title of this address, and possibly even from the purpose of this series of lectures, in talking about physical science in the education of the future doctor: but if biophysics is to make its contribution to medicine it is necessary that most doctors should have some idea at least of what it is about, while some doctors should have a pretty good idea. The ideas and methods of physics and of physical chemistry are being applied today, and will increasingly be applied, not only directly to physical medicine and radiology, but to neurology, to the study of circulation, respiration and excretion, and of the adjustment of the body to abnormal conditions of life and work. At longer range, moreover, they will be aimed at the fundamental problems of minute structure and organization, of the physical basis of growth and inheritance, of the ordered and organized sequence of chemical reactions in vital processes, of the means by which energy is supplied and directed to

vital ends. It is inevitable today that most of the discoveries in such fields will be made by people who have not passed through the gateway of medicine: but the greatest good comes from mixing people up, so that research and development (to use an industrial term) in promising fields can be guided to practical ends. That implies that those whose ends are practical (and the aims of medicine, like those of engineering, are practical, however great their intellectual content) should be on terms of familiar equality with those whose moving force is scientific curiosity. It is necessary that each should know what the other is talking and thinking about. The physicists and the chemists must be acquainted with biology, the biologists (including the medical people) must know some physics and chemistry. The chief answer, then, to the query *Why Biophysics?* is that the recognition of biophysics as a special subject of study will emphasize the fact, and I think it is a fact, that the future of biology and medicine will increasingly require the application of physical and physico-chemical ideas and methods. To give biophysics a name and personality, to endow it with a few centres where it can be specially practised, to realize that its recognition implies much more than just mixing up biologists and physicists (good as that is), will draw in recruits to a science that in twenty years or so may have the same importance to biology and medicine as biochemistry has come to have today. I am content myself to have been a physiologist, but that was luck because Walter Morley Fletcher was my tutor at Cambridge. Others of like tastes, but without such good fortune, may not find their way in at all unless the road is open and given a distinctive name.

II

The New Look in Virus Research

C. H. ANDREWES

FASHIONS in the techniques of virus research change from time to time just as do fashions in dress. A Martian virologist visiting the earth every five years or so would, on his successive visits, find us doing things different from those which were occupying us on his last call. In fact, new techniques are being constantly introduced and developed and these lead to regular extensions of the frontiers of knowledge. Dare I suggest that the New Look which virus research is periodically showing, is more worth while than the odd New Looks which we perceive in human fashions?

Let us see how we have been able to learn more and more from new techniques I embarked upon virus research about thirty years ago. Our methods were few. The rabbit was the main object of our study Herpes simplex, vaccinia, rabies and a few other viruses were inoculated into rabbits intracerebrally, vaccinia and other viruses into its skin and its testes, and we learnt a lot of things, some of which we have since had to unlearn. For example, the French school wrote freely on tropisms of viruses for tissues derived from the different germinal layers—ideas which we hear little of today. By 1930 the Medical Research Council was able to publish a volume on viruses of over 500 pages in its *System of Bacteriology*. Much was then known of formation of specific inclusion bodies by various viruses, their sizes were beginning to be determined by the aid of Elford's graded collodion membranes; some viruses had been grown in tissue culture. But many viruses could only be studied in their often inconvenient natural hosts—dogs, sheep, cattle and so on.

The mouse, now the virologists' favourite animal, is hardly mentioned in the MRC volume. I wrote a chapter on 'virus diseases of rabbits and guinea pigs', but there was nothing about diseases of laboratory mice, for none was known. Nowadays, we know of only too many of the native virus diseases of mice and they confuse our researches at every step.

Though both had been used before, it was in the early 1930s that mice and eggs may be said to have swum almost simultaneously into the virologist's ken; and they have proved invaluable ever since. Webster and Fite (1933) transmitted St. Louis encephalitis to mice by intracerebral inoculation. That was the first of the arthropod-borne encephalitis viruses to be seriously studied, and the mouse made that possible. Now we can read volumes and volumes upon Eastern, Western and Venezuelan encephalitis, Japanese B and Murray Valley encephalitis, louping-ill, dengue, yellow fever and a whole host of tropical viruses isolated from man, monkey or mosquito and detected by the effects produced when they grow in the brains of mice. So too for viruses of veterinary interest such as African horse-sickness and blue-tongue. Nor is it only the arthropod-borne viruses which grow in mouse brains; fowl plague, some strains of influenza and poliomyelitis will do so too. These studies soon made it clear that mice have their own natural brain parasites among viruses liable to be picked up and activated by brain to brain passage.

In 1934 influenza A virus, which in the previous year had been shown to infect ferrets, was adapted to kill mice when inoculated intranasally under an anaesthetic (Andrewes, Laidlaw and Smith, 1934), and a method became available for economical titration of that virus and corresponding antisera. The mouse's lung has, however, not proved such a useful medium as its brain, for only a few other viruses can be conveniently studied by this technique, apart from the native pneumotropic mouse-viruses which, as with the intracerebral work, soon came to light to confuse matters.

In 1931 Goodpasture adapted to virology the technique of cultivation in fertile developing hens' eggs, and soon afterwards Burnet extended and developed the method (cf. Beveridge and

Burnet, 1946). It was soon found that the vast majority of viruses would grow by one or other method in eggs, either after inoculation on to the chorio-allantoic membrane or into the amniotic, allantoic or yolk-containing cavities or into the body of the embryo itself. Amnion, allantois and yolk-sac each had its advantages for special purposes. Influenza viruses freshly isolated from man go best in the amniotic cavity; after adaptation, however, they grow to high titre in the allantoic cavity, inoculation of which is a much simpler technique: further, the allantois provides a more plentiful source of virus-containing fluid, available in the case of influenza as a source-material for vaccines. The yolk-sac is best for some viruses and especially for rickettsiae; while for rabies and yellow fever the body of the embryo provides the best material for making vaccine.

A consequence of this new advance was Hirst's discovery (1941) that allantoic fluids containing influenza virus in high titre would agglutinate fowl red cells. This led to the studies of Burnet and his colleagues on the receptors whereon viruses of the 'flu group attach themselves to cells. Another very new look was to be seen on the face of virus research.

Since the war a number of new techniques and ideas have come into virus research. Since these give the subject its current new look, I will deal with them in more detail. They include the use of the suckling white mouse for virus studies, the cultivation of viruses in growing human tissues, the conception of latent viruses, the application to animal viruses of ideas derived from study of 'phages or bacterial viruses, and the evidence for recombination or hybridization of viruses.

We may note in passing that very little has come from the use of 'unusual' animal species. The ferret played an important part in research on dog distemper and influenza. Adaptation of poliomyelitis to a rodent was first achieved with the help of cotton-rats. Golden hamsters have proved useful in several ways. On the whole, however, apart from the necessity of using natural hosts of a virus, workers have got along pretty well with rabbits, guinea-pigs, mice and eggs, and some monkeys.

Use of Suckling Mice

Coxsackie viruses. Dalldorf and Sickles (1948) isolated in suckling mice a virus from filtrates of faeces of children with paralysis; this differed from poliomyelitis and since the children lived in the village of Coxsackie, the virus was provisionally called Coxsackie, sometimes abbreviated to C. Though the name was only meant to be provisional, it has stuck. Coxsackie viruses resemble polio virus in being tough, resistant to ether, and in being amongst the smaller ones known. Numerous serological types of Coxsackie viruses have now been recognized. Two main features of them are that they can be found in faecal filtrates (also in throat washings) and that they produce infection in mice of a few days old, but not in older mice. Obviously these are not likely to be satisfactory criteria, and indeed viruses are being found which attack particularly baby mice and are clearly unrelated to the Coxsackies.

There is gradually emerging clear evidence that at least some types of Coxsackie viruses are related to human disease· they can be placed in two main groups. The group A viruses—of at least ten serological types—have been recovered from patients suffering from mild fever and sore throat with lesions on the fauces, of a type described as herpangina. Convalescents from infection develop antibodies against the virus. Viruses of this, A, group, produce paralysis in baby mice, associated with production of acute myositis. Viruses of a second group, B, seem to be associated rather with epidemic pleurodynia or Bornholm disease; these may cause myositis in baby mice but also lesions of the central nervous system, fat and viscera. Group B viruses may be rather larger than those of group A. Coxsackie viruses have also been isolated from cases of aseptic meningitis in man and from cases diagnosed as 'non-paralytic polio'. Indeed a confusing relationship to poliomyelitis affords a particular reason for their importance. They may produce clinically similar symptoms, and they are isolated from the same source, faeces, by similar techniques They tend to behave similarly epidemiologically and indeed polio virus and C viruses have on numerous occasions been recovered from the same materials. No convincing evidence exists that eithe

Burnet, 1946). It was soon found that the vast majority of viruses would grow by one or other method in eggs, either after inoculation on to the chorio-allantoic membrane or into the amniotic, allantoic or yolk-containing cavities or into the body of the embryo itself. Amnion, allantois and yolk-sac each had its advantages for special purposes. Influenza viruses freshly isolated from man go best in the amniotic cavity; after adaptation, however, they grow to high titre in the allantoic cavity, inoculation of which is a much simpler technique: further, the allantois provides a more plentiful source of virus-containing fluid, available in the case of influenza as a source-material for vaccines. The yolk-sac is best for some viruses and especially for rickettsiae; while for rabies and yellow fever the body of the embryo provides the best material for making vaccine.

A consequence of this new advance was Hirst's discovery (1941) that allantoic fluids containing influenza virus in high titre would agglutinate fowl red cells. This led to the studies of Burnet and his colleagues on the receptors whereon viruses of the 'flu group attach themselves to cells. Another very new look was to be seen on the face of virus research.

Since the war a number of new techniques and ideas have come into virus research. Since these give the subject its current new look, I will deal with them in more detail. They include the use of the suckling white mouse for virus studies, the cultivation of viruses in growing human tissues, the conception of latent viruses, the application to animal viruses of ideas derived from study of 'phages or bacterial viruses, and the evidence for recombination or hybridization of viruses.

We may note in passing that very little has come from the use of 'unusual' animal species. The ferret played an important part in research on dog distemper and influenza. Adaptation of poliomyelitis to a rodent was first achieved with the help of cotton-rats. Golden hamsters have proved useful in several ways. On the whole, however, apart from the necessity of using natural hosts of a virus, workers have got along pretty well with rabbits, guinea-pigs, mice and eggs, and some monkeys.

though there had really been infection of C_3H cells, though the disease was 'conditioned'—that is, it only showed itself in later life when other factors were just right. The finding recalls the facts about the Bittner breast cancer virus of mice: this also produces its effects only late in life under the influence of an appropriate hormonal background. Gross's results with mouse-leukaemia remain, for the most part unconfirmed, but those working on the subject are not at present inclined to dismiss them.

Use of Human and other Tissue Cultures

We have perhaps come near the end of the honeymoon period of virus research, happy days when one could hope to reveal a new virus by transmitting infection to a rabbit, a mouse or even an egg. Most animal-pathogenic viruses will attack one or other of these: but there remains a hard core of the strictly species-specific ones and of these we, as doctors, are particularly interested in those human viruses which insist upon a human pabulum. For them we can use volunteers, but this method is available only for very harmless viruses such as the common cold. Now, for the others, people are beginning to use *in vitro* cultures of human tissues.

Viruses have been grown in tissue cultures for thirty years or more but until lately the dividends yielded by this practice have been of theoretical interest only and not too great at that. Enders, Weller and Robbins (1949) brought a big change when they cultivated poliomyelitis virus in human tissues other than those from the nervous system, and then proceeded to show that the cytopathogenic or destructive effects produced in cells of the cultures could be used as the basis of simple naked-eye quantitative methods. Today we all know that we can test falling dilutions of virus-containing material in tissue cultures and estimate its virus content by the cytopathogenic effects observed that we can similarly estimate antibody titres by observing the inhibition of the effects of the virus, that we can apply sera known to be of types I, II or III to serological typing of polio viruses; and finally that we can use tissue cultures as a means of producing large quantities of virus for vaccines. Human tissue cultures were used in the earlier studies along

of the other in man, for better or for worse. There is no doubt that C viruses can be recovered from many specimens of human faeces without any association with illness.

The Coxsackie work is important for two reasons. It has revealed the existence of a hitherto unsuspected group of pathogenic viruses, of importance in human medicine. Secondly, it has given us what amounts to a very useful new experimental animal, the suckling mouse. It has long been known that for many viruses, the younger the animal the greater the susceptibility: for instance, many viruses grow in developing eggs, though they will not do so in hatched chicks. Also many neurotropic viruses will infect young mice when injected intraperitoneally, though they can attack the older ones only when given directly into the brain. Now the idea has been carried to its logical conclusion by infecting mice almost as soon as they are born. The baby mouse is duly proving useful in other fields of virus research. It is a most useful animal for studying foot-and-mouth disease, in which it can be used for titrating virus and antisera and as a source of virus for making vaccine (Skinner, 1951). An important disease of sheep in Africa and North America, blue-tongue, produces symptoms in baby mice, though causing silent infections in older ones. Some of the arthropod-borne viruses from Africa must be isolated in baby mice. Instances could be multiplied.

A most exciting suggestion is that leukaemia in inbred mice—a disease otherwise behaving like other mammalian leukaemias—may be transmissible by means of filtrates provided inoculation is into baby mice preferably less than one day old. Gross (1951), who made these observations, used a strain of mice with spontaneous leukaemia, Ak, and made the inoculations into another line of inbred mice, C_3H. Leukaemic cells of Ak mice would produce leukaemia in baby C_3H mice; the leukaemia came on quickly and was readily transmissible in older Ak mice, hardly at all in C_3H. That is, it was only a question of survival of the Ak leukaemic cells for a time in the C_3H mice. But when he used filtrates, the inoculated C_3H only developed leukaemia after something like a year and this leukaemia was then transmissible to C_3H mice not Ak mice. In other words, it looked as

in series through various kinds of human tissue cultures. They, and antibodies engendered by them could be titrated by methods similar to those in use in polio research.

Very shortly after these findings, Hilleman and Werner (1954) described the isolation of viruses from throat washings from cases of epidemic upper respiratory infection. These were likewise recognized by the changes induced in human tissue cultures and, in short, are clearly of the same family as Huebner's adenoid viruses. These viruses, at least in part, are no longer 'in search of a disease' but seem to be associated with epidemics of pharyngitis and so forth. I say advisedly 'in part' for these new viruses now seem to belong to at least six serological types. Types 5 and 6 have only turned up on a few occasions and I shall not discuss them. Types 1 and 2 have been grown from adenoids or tonsils and, in Huebner's view, may cause disease in children but are pretty well non-pathogenic for adults. I glide over the question of whether the adenoids in question were or were not normal, for the point of course arises of whether an E.N.T. surgeon ever removes normal adenoids or tonsils. It would certainly be nice to know whether any effects of these viruses caused the condition for which the operation was done, or whether they will be equally present in all adenoids.

Types 3 and 4 are the ones associated with epidemic disease. The Americans call this infection A.R.D. which is short for acute undifferentiated respiratory disease. In this country it has been called febrile catarrh: it is probably the infection which is especially common in Army recruits, and at the beginning of the spring term in boarding-schools. Fever, pharyngitis and laryngitis are common. It may be confused with influenza, but has a more insidious onset, clinically it differs in that local symptoms—sore throat, cough and so on—are relatively commoner, and general ones such as prostration, aches and pains relatively less so. Huebner records conjunctivitis as a common symptom in some of his cases; in fact he suggests calling these viruses the A.P C. viruses. A.P C stands for adenoid-pharyngeal-conjunctival and implies, though it well might, no suggestion as to a suitable combination of drugs for its relief. The

these lines, but it has since been found that those of monkeys, especially from kidneys, can be used just as readily.

In the case of measles, also, tissues from man and monkey have been shown capable of supporting virus growth. Enders and Peebles (1954), who describe this, found peculiar cytopathogenic effects, particularly the running together of cells into syncytial masses. Their tissue cultures contained an agent capable of fixing complement in the presence of convalescent human serum. It seems likely, then, that we are on the verge of being able to study this virus, also, in a quantitative manner. The susceptibility of monkeys to actual infection with measles virus has always been so erratic that progress has been halting. Anderson (1954) has lately reported from Australia that the virus of rubella can be similarly grown; but it is likely that this virus may be less amenable than ordinary measles.

Weller (1953), one of Enders' colleagues, has been successful in growing varicella virus—and here human tissues were necessary. He had the advantage that a characteristic intranuclear inclusion body is associated with chicken-pox lesions, so he had something definite to look for. Such inclusions were found in infected cultures, in which foci of infection seemed to spread centrifugally to adjacent cells, virus was not readily demonstrable in the fluid phase of the cultures.

In Africa, workers seeking for yellow fever virus in mosquitoes, have brought to light other viruses, pathogenic when injected into mouse brains and possibly, but only possibly, important as causes of human illness. So, too, workers injecting baby mice with faecal filtrates or infecting human tissue cultures with this and that, have uncovered new viruses of still undetermined importance. They have been called orphan viruses or viruses-in-search-of-a-disease.

Huebner and his colleagues (1954) discovered new viruses of such a kind when making cultures of adenoids and tonsils removed at operation. Cell cultures of adenoids often grew out well, but at other times a granular degeneration soon set in. It was found that from such cultures, agents could be recovered which induced similar changes in previously normal cultures; these agents had the properties of viruses and were transmissible

blind alley. Many latent infections are not irrevocably latent. Thus latent herpes infection of the human mouth and lips may

relationship of phage and bacterium in a lysogenic organism. The phage—or, if you like, prophage—seems to be associated with the genetic make-up of the bacterium, and to divide *pari passu* with the chromosomes—or their equivalent—in the host. One would not know it was there unless one applied what Lwoff *et al.* (1950) call a shock or inducing stimulus (such as ultra-violet irradiation) and thus upset an equilibrium with resulting production of an actively lytic phage. It is entirely logical to believe that in the course of evolution many viruses and their hosts have become blended in a closer and closer union, the infection becoming more and more completely latent (Andrewes, 1952). When blending is complete, we can hardly hope to recognize the virus' existence. The only latent viruses we can recognize are the ones in which the union has stopped short of irreversibility. One can argue—and I have done so previously—that the only logical basis for belief in a virus theory of cancer is to conceive of a closely integrated virus-cell association, disturbance of which permits the virus to act as a continuing stimulus to the cell's unruly multiplication. It need not follow that the result of the disturbance need be liberation of virus in a state able to infect normal cells. In other words a tumour caused by a virus need not be transmissible by means of filtrates. Gross has written of 'vertical transmission' of a virus from parent to offspring as an alternative method to the more usual one of horizontal transmission from an infected host to its normal neighbour.

I will mention briefly one other example of a latent virus infection. These infections commonly reveal themselves in laboratory animals when one makes serial passages of organ-suspensions from one animal to another. Thus neurotropic viruses turn up in mice when brain-suspensions are passaged intracerebrally; and pneumotropic viruses appear when passages are made of lung-suspensions by intranasal inoculation.

types are similar when tested by complement-fixation—a fact which serves to place them all in one group: it is neutralization in tissue cultures which differentiates them.

We are thus, doubtless, at the beginnings of an inquiry into another group of viruses, possibly just as important as the Coxsackie viruses or even more so. There seems good reason to believe that they are actually associated with febrile catarrhs, for convalescents do develop antibodies against them. But of their importance in medicine, their possible relation to sporadic cases of respiratory infection and so on, we at present know very little. Those of us who have been working for years on influenza and the common cold have always known that there existed a big group of infections neither colds nor 'flu; it is very gratifying to have at hand a new tool with which further to be able to sort these things out.

Doubtless yet more peculiarly human viruses remain to be revealed by the very logical technique of human tissue culture. Two years ago one strain of the common cold was grown by my colleagues in such a way for ten serial passages: but difficulties have been met with, and further progress cannot now be reported. Infectious hepatitis is the outstanding example of an important virus disease of man, where neither adaptation to animals nor cultivation in tissues or by other means looks, at the moment, very promising.

Latent Viruses

The idea that many virus infections may be latent is not a new one, rather one which is increasingly gaining recognition. The parasite and host which have long existed together have learnt to tolerate one another. Thus many virus infections exist unobserved in their natural hosts. Accidental transfer to a strange host—as by an insect—may give rise to a striking disease which is yet wholly irrelevant to the normal natural history of the parasite. Some of the encephalitis viruses, in North America and elsewhere, exist as symptomless infections in birds, and are carried by mosquitoes. Occasionally an infected mosquito bites a man or a horse and produces fatal encephalitis. Such an incident means, to the virus, merely that it has strayed up a

or other bits of vertebrate cells could grow in insects or vice versa. Further, some plant viruses will multiply in insects—which could lead on the errant-gene hypothesis to some very remarkable conclusions. You will gather from these remarks that my own bias is strongly towards the more orthodox view that viruses are organisms.

But if they are, what sort of organisms are they? Do they multiply, as bacteria do, by binary fission. Here is one of the most fascinating fields of current virus research. It may well turn out that there is a fundamental difference between bacteria and viruses as regards how they multiply. Taxonomists are discussing nowadays whether viruses should come under the bacteriological code of nomenclature. It is conceivable that we ought to classify living things first as viruses and non-viruses and then consider, as to the non-viruses, the minor matter of whether they are animals or vegetables.

It seems that when a virus enters and infects a cell, it loses its identity for a time: we cannot recognize it as the virus particle we have previously known: we cannot demonstrate its infectivity for fresh cells or fresh hosts. Only after a period in what is called the 'eclipse phase', does it reappear as the infective particle with familiar morphology. I like to think of the inert tough particle which we handle in the laboratory and call 'virus' as something equivalent to the spore—the phase in which the virus gets about and finds a new host (Andrewes, 1952). The actively multiplying virus in the eclipse phase can be regarded as in the vegetative part of its life-cycle, and this is the real virus which is multiplying and doing things to its host. A clue to what is happening is furnished by studies of phages or bacterial viruses One of the surprising things the electron micrograph taught us was that many phages have heads and tails. These can be separated by mechanical means. The interior of the head seems to consist of nucleoprotein of the DNA type; the outer shell of the head plus the tail consists of phosphorus-free protein. The former consists of the essential part of the phage responsible for its genetic continuity the tail and the shell are merely part of the mechanism for getting it inside a susceptible organism. A phage attaches to a bacterium tail

My colleagues and I at the National Institute for Medical Research have lately studied a hepatitis virus in mice. This causes, in weaned mice, rather trivial, non-fatal lesions. Another parasite, *Eperythrozoon coccoides*, occurs in many stocks of mice, causing no particular trouble. It sits on red cells in the bloodstream, being visible as little rings. In the presence of this normally harmless parasite, the normally harmless hepatitis virus produces a rapidly fatal hepatitis (Niven *et al.*, 1952). Now it seems that this virus exists in many, perhaps most, stocks of mice in a latent form. But one cannot activate it by serial intraperitoneal passages of liver-suspensions as one might expect from analogy with the neurotropic and pneumotropic viruses. But if one inserts into the passage series this other parasite, *E. coccoides*, something happens to exalt the activity of the virus and up comes a fatal hepatitis. The *E. coccoides* seems to act as an agent upsetting the virus-host equilibrium and turning a latent virus into an active one, just as Lwoff's ultra-violet light induced the prophage to become lytic phage.

During this lecture I have been progressing from the more practical to the more academic side of virus research, showing first how new techniques helped us to reveal and study yet more viruses attacking man and latterly considering the rather more theoretical question of latent viruses. We will now become still more academic and consider briefly what viruses are and how they multiply.

Nobody argues any longer as to whether viruses are alive or dead. The fact that some plant viruses can be crystallized still excites wonder in the lay mind, but not amongst those who have thought more deeply Viruses are undoubtedly alive the ground of the argument has shifted. Are they highly specialized parasites derived, perhaps, from larger organisms, or can they be fragments of a cell, perhaps clumps of genes, which become pathogenic as well as self-perpetuating if transferred to the cell of another host? I shall not discuss this further since we cannot devise crucial experiments to determine which view is correct. I would, however, draw your attention to the fact that a good many animal viruses can multiply both within the cells of vertebrates and insects—it would be very surprising if genes

They have done much more work since then and have used a great number of different markers with the aid of which to trace the reassortment of properties which can occur when cells are infected simultaneously with several viruses. Most of the work has been carried out, not in mice, but in infected eggs. If we look upon viruses as very primitive things instead of degenerate parasites, it is tempting to consider these recombinations as representing the earliest dawnings of sex-consciousness in the primeval ooze.

It is not, however, all that simple. When two viruses infect a cell, there may emerge viruses with mixed characters, and yet this mixed virus may fail to breed true. Two serologically distinct influenza viruses may be grown in eggs: let us call them G and H, which are amongst the few letters carrying no other implications that I know of in the influenza field. If both are present in infected egg-fluids, haemagglutination by this fluid will be inhibited by neither anti-G antiserum alone nor by anti-H, for either way there will be particles present which are not neutralized and are thus able to agglutinate red cells Only a combination of anti-G and anti-H fluids will stop the haemagglutination. Hirst and Gotlieb (1953), however, were able to grow in mixedly infected eggs viruses which were neutralized by either anti-G or by anti-H sera; in other words they were composite GH viruses, reacting with either antibody. In general, these mixed viruses did not breed true. It seems possible that if influenza virus multiplication occurs on the model of phage, the essential nucleoprotein manufacture goes on first, and that determines the genetic character of the product, the genotype, or if you prefer, the chassis. At the later stage, that of assembly of the parts of the body, and the accessories, there is built in whatever comes handy and one may end with a product or phenotype with most miscellaneous and ill-assorted characters It is even possible to obtain a 'flu virus which is phenotypically a mixture of viruses A and B, but genotypically it is one or the other—a true hybrid has not been obtained

Burnet and Lind (1954) suggest that gradual changes in virulence of a virus depend upon association of the 'chassis' with varying numbers of virulence genes. They find that

first, and then it acts like a little syringe; the nucleoprotein is injected, apparently through the tail, into the bacterium, all the shell-tail apparatus being regarded as expendable and left outside. Once inside the bacterium, the nucleoprotein multiplies as such at the expense of the bacterial contents and of the fresh material accruing thereto from outside so long as bacterial metabolism continues. Only at a late stage in the proceedings does the phage begin to form more of the shell-tail outfit which has to be fashioned around the nucleoprotein to constitute once more a complete phage-particle capable of infecting new cells.

There is much speculation as to what is happening in the early stages; bacteria may be simultaneously infected with several phages and there may be constituted within them a nucleoprotein pool which, because of its diverse origin is not homogeneous. In the outcome, phage particles may be manufactured, having mixtures of the characters of the original particles—what are called recombinants, or, if you like, phage hybrids. These may breed true on further propagation.

Burnet and his colleagues (1951) have followed up this idea and have obtained evidence for similar happenings with influenza viruses. 'Interference' is a well-known phenomenon to virus workers. one virus, not itself so pathogenic, may interfere with the activity of a related or unrelated more vicious virus and inhibit its effects. A variant of influenza A virus is known, neurotropic WS (NWS), capable of growing in mouse brains on intracerebral injection, and killing the mice It is serologically readily distinguished from other influenza A strains such as that causing swine influenza These fail to multiply progressively in mouse brains. Burnet injected mice intracerebrally with a little neurotropic WS plus a lot of swine influenza, but not enough of the latter to suppress the NWS completely. Brains of the mice were harvested and the virus cultivated in eggs along with enough antiserum against NWS to discourage that virus. By balancing things just right they were able to end up with a 'recombinant' virus having the power to grow intracerebrally in mice and to kill them, but having the antigenic characters of swine influenza Such a recombinant bred true on passage.

HUEBNER, R. J., ROWE, W. P., WARD, T. G., PARROTT, R. H. and BELL, J. A. (1954). *New Engl. J. Med.* 251, 1077.
LWOFF, A., SIMINOVITCH, L. and KJELDGAARD, N. (1950). *Ann. Inst. Past.* 79, 815.
NIVEN, JANET S. F., DICK, G. W. A., GLEDHILL, A. W. and ANDREWES, C. H. (1952). *Lancet*, ii, 1061.
SKINNER, H. H. (1951). *Proc. roy. Soc. Med.* 44, 1091.
WEBSTER, L. T. and FITE, G. L. (1933). *Science*, 78, 463.
WELLER, T. H. (1953). *Proc. Soc. exp. Biol., N.Y.* 83, 340.

recombinants of strains with high and low virulence for mouse brain or mouse lung may show virulence of intermediate grades.

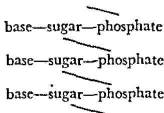
All this brings many new ideas into virus research. We may soon have much better insight into the mechanisms underlying virus variation and adaptation. There are even hopes that we may one day be in a position to control events by designing and obtaining tailor-made viruses for purposes of immunization.

Still, I have not had space to touch on many of the other new looks worn by virus research. The plant virologists have been as busy as those studying animal infections. I have not discussed the work of Burnet and others on the mucoprotein receptors and their relation to the attack of viruses upon cells; nor to all the work done by electron microscopists on morphological aspects of viral multiplication. I have not mentioned 'incomplete virus' nor even one of my own special interests, the changes in influenza virus in their relation to epidemiology. There are as many facets in modern virus research as there are in the compound eye of a butterfly, and I could only tell you of such 'new looks' as seemed most likely to titillate your interest and curiosity.

REFERENCES

ANDERSON, S G (1954) *Lancet*, ii, 1107
ANDREWES, C H. (1952) *Proc. roy Soc*, B, 139, 313
ANDREWES, C. H, LAIDLAW, P P. and SMITH, W (1934) *Lancet*, ii, 859
BEVERIDGE, W B and BURNET, F. M. (1946) *Spec Rep Ser med Res. Coun, Lond.*, No 256.
BURNET, F M and LIND, P E. (1951) *J. gen. Microbiol* 5, 59, 67
BURNET, F M and LIND, P. E (1954). *Nature, Lond* 173, 627.
DALLDORF, G and SICKLES, G M. (1948) *Science*, 108, 61
ENDERS, J F. and PEEBLES, T C (1954). *Proc Soc exp. Biol, N.Y* 85, 277.
ENDERS, J. F, WELLER, T. H. and ROBBINS, F. C (1949) *Science*, 109, 85
GROSS, L (1951). *Proc Soc exp Biol, N Y* 76, 27
HILLEMAN, M. R and WERNER, J H (1954) *Proc. Soc. exp Biol, N Y.* 85, 183
HIRST, G K (1941) *Science*, 94, 22.
HIRST, G. K. and GOTLIEB, T (1953). *J. exp Med.* 98, 41, 53.

Two series of nucleic acids are known to exist, the *ribonucleic acids* (RNA) in which the sugar is D-ribose and the *deoxyribonucleic acids* (DNA) in which the sugar is D-2-deoxyribose. They also differ in their complements of bases as shown in Table 1. [illegible] *next nucleotide so as to form a long chain of nucleotides thus:*



The alternating sugar and phosphate residues form the backbone of the molecule from which the bases project to the side like flat shelves. In RNA side chains may be attached at certain of the sugar residues, although this is not yet certain, but in DNA the structure is slightly more complex. Chargaff (1950)

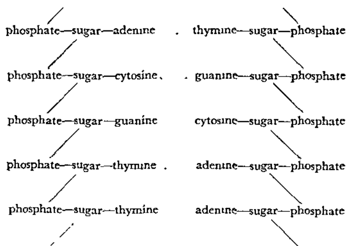


FIG. 1 Diagrammatic representation of part of a hypothetical polynucleotide chain in DNA.

III

Nucleoproteins in Cell Structure

J. N. DAVIDSON

ALTHOUGH the nucleic acids have been known to biochemists since the days of Miescher (1844–95) they have until recently been regarded as rather complex structural units of the cell with no well-defined specific biological properties Within the last ten or fifteen years, however, intense interest has been shown in these substances both on the chemical side on account of their unusual chemical structure and on the biological side on account of their remarkable biological activity (Chargaff and Davidson, 1955) In this survey an attempt is made to review some of the recent developments in this field.

Chemical Structure

Nucleic acids may be regarded as being built up from units known as *nucleotides*, each nucleotide consisting of a purine or pyrimidine base, a pentose sugar and phosphoric acid thus:

base—sugar—phosphate.

Table 1 Components of nucleic acids

	RNA	DNA
Purine bases	Adenine Guanine	Adenine Guanine
Pyrimidine bases	Cytosine Uracil	Cytosine Thymine (methyluracil) 5-Methyl cytosine[1] Hydroxymethyl cytosine[2]
Sugar	D-Ribose	D-2-Deoxyribose
Inorganic component	Phosphoric acid	Phosphoric acid

[1] In small amounts
[2] In certain DNA's from bacteriophage only

many DNA's (Table 2). Those from different sources differ in their relative proportions of purine and pyrimidine bases although there is evidence that the DNA's from the different tissues of any one species have very similar compositions in terms of the molar properties of bases. The type of DNA may in fact be characteristic of the species.

Apart from variations in the relative proportions of bases, different RNA's or DNA's may differ from each other also in the sequence in which the different bases occur along the polynucleotide chain. To this point we shall return later.

TABLE 2 Molar proportions of bases related to adenine as 10 in RNA and DNA from different sources

(Data from various authors)

Source	Adenine	Guanine	Cytosine	Uracil	Thymine	5-methyl cytosine
			RNA			
Human liver	10	16 9	14 7	10 8	—	—
Yeast	10	12 1	7 8	9 0	—	—
Tobacco mosaic virus	10	9 0	6 1	9 1	—	—
			DNA			
Human thymus	10	6 4	6 4	—	9 5	0 5
Human liver	10	6 5	6 6	—	10 0	—
Wheat germ	10	8 9	6·5	—	10 2	2 2
Mb tuberculosis (avian)	10	23 1	23 4	—	9·7	0
E. coli phage T5	10	6 4	6 4	—	10 1	0
E coli phage T2r	10	5 7	5 3[1]	—	10 0	0

[1] All as hydroxymethyl cytosine

The localization of the nucleic acids in the cell has been worked out both by the use of histochemical tests on tissue sections and by the chemical analysis of isolated cell components. For example it is known from the results of the Feulgen histochemical test and from the analysis of isolated cell nuclei that DNA is found exclusively in the cell nucleus where it exists in combination with protein as a nucleoprotein complex which the histologists have for a long time known under the name of *chromatin*.

has shown that in DNA the number of adenine residues in the molecule is approximately equal to the number of thymine residues and the number of guanine to the number of cytosine residues. Consequently if the different purines and pyrimidines form a definite sequence along the chain and if we place a second chain opposite the first so that its purines come opposite the pyrimidines of the first and conversely, we obtain a structure such as shown in Figure 1. The purines of the one chain may now link up in pairs with the pyrimidines of the other chain through hydrogen bonds, forming, as it were, the rungs of a ladder in which adenine links up with thymine and guanine with cytosine. The sequence of bases for one chain has been drawn completely at random in Figure 1 but once it has been fixed the sequence of the other chain is automatically decided also.



FIG 2 Diagrammatic representation of the Watson and Crick (1953) structure of DNA.

And if the resulting structure is coiled into a spiral to form a structure resembling not a ladder but a spiral staircase the treads of which are formed by a purine from one side meeting a pyrimidine from the other side, we get a very crude representation of the DNA molecule.

The suggestion that the DNA molecule has an architecture such as has been described was put forward two years ago by Watson and Crick (1953) and supported by Wilkins *et al.* (1953) and by Franklin and Gosling (1953) as the result of some very beautiful X-ray diffraction studies. Their picture of the molecule is illustrated in Figure 2 as two helical phosphate sugar chains winding round the same axis with the chains running in opposite directions and held together by hydrogen bonding between complementary pairs of purine and pyrimidine bases in such a way that one helix is the complement of the other

It must be emphasized that in nature many RNA's exist and

transforming principle is glycine which has been shown to be derived from the breakdown of adenine.

This is only one example—the first to be discovered and the most striking—of bacterial transformation induced by DNA. Others are known affecting the pneumococcus, *Haemophilus influenzae*, *E. coli* and the meningococcus (Hotchkiss, 1955). More than twenty different capsular transformations are now known, involving at least fifteen different polysaccharide antigens, and at least thirty biochemically distinct characters have been introduced *in vitro* by bacterial DNA into living cells of the homologous species. A few of these transformations are indicated in Table 3.

TABLE 3. Some characters transferred in bacterial transformations brought about by DNA preparations *in vitro*

Capsular antigens	
	D. pneumoniae
	H. influenzae
	N meningitides
	E. coli
Drug resistance	
Penicillin	*D. pneumoniae*
Streptomycin	*D pneumoniae*
Streptomycin	*H. influenzae*
Sulphanilamide	*D pneumoniae*

Some of the most recent examples are from the very beautiful work of Hotchkiss (1955) on drug resistance and antibiotic resistance. In any culture of pneumococci, for example, occasional mutants arise which are resistant to penicillin. If these are picked out and cultured and used as the source of DNA, the DNA so obtained, when added to the medium in which penicillin-sensitive organisms are growing, will induce the property of penicillin resistance. This property once induced is transmitted to succeeding generations.

The same mechanism appears to hold for streptomycin resistance and for sulphonamide resistance but it must be emphasized that in all cases of bacterial transformation only a very small fraction, usually less than 1 per cent, of the cells, is permanently modified. Nevertheless the results are sufficiently

RNA on the other hand has a much wider distribution in the cell. A small amount is found in the cell nucleus but most of it is found in the cytoplasm, partly in the large and small cytoplasmic inclusions, and partly in the form of the non-particulate soluble nucleoprotein of the cell sap.

The Biological Properties of DNA

From the biological point of view it is obviously a matter of very great interest to know just what part is played by the nucleic acids in the economy of the cell. Here our knowledge is fragmentary and our views are speculative but some definite evidence of considerable interest has come to light in recent years. The first evidence relates to the genetic functioning of DNA and the original observation on which much subsequent work has been based was made in 1944 when Avery, McLeod and McCarty showed that DNA extracted from an encapsulated 'smooth' strain of pneumococcus could, on addition to the culture medium, transform unencapsulated 'rough' cells into the fully encapsulated smooth type. Such transformations of bacterial type could be brought about in rough strains which had never been known to undergo spontaneous mutation to the smooth type and the smooth cells so developed could propagate indefinitely as a smooth type which would continue to produce DNA with the same capabilities. The pneumococcal DNA had therefore initiated its own reduplication as well as inducing the specific inheritable property of capsule synthesis. These are two functions usually associated with the genes, and it is clearly important to verify that the chemical substance responsible is DNA and DNA alone. It might be argued for instance that the transforming power of DNA is due to the presence in the purified DNA of traces of protein or carbohydrate and this question has been the subject of careful scrutiny. The DNA of purified transforming principle acts in very minute amounts, it contains neither chemically nor serologically detectable protein, and it is not inactivated by proteolytic enzymes such as trypsin or chymotrypsin. On the other hand it is inactivated by the enzyme deoxyribonuclease which specifically attacks DNA. Moreover, the only amino-acid present in hydrolysates of the

Crampton *et al.*, 1954) by using modern techniques have separated the different DNA's of calf thymus nuclei into groups which differ from each other slightly in composition as shown by the relative molar proportions of bases.

Brown (1955) has even gone a stage further and has fractionated the DNA from streptomycin-resistant pneumococci into a series of fractions which differ not only in chemical composition but also in their ability to induce streptomycin resistance in sensitive organisms.

It is of course clear that this evidence of heterogeneity within the species must somehow be reconciled with the earlier evidence that *the overall composition of* DNA *is characteristic of* the species, but two possible solutions may at once be suggested. (1) The different DNA molecules in any one kind of nucleus may all contain the same proportions of bases but may have different sequences of bases along the polynucleotide chain; or (2) the DNA complement of any one nucleus may be a mixture of DNA molecules of different composition as regards the proportion of bases but the same mixture of DNA molecules may be present in all nuclei of the one species.

Clearly these two possibilities can exist side by side, and the evidence of heterogeneity in composition obtained by Brown and Watson and by Chargaff shows that the second possibility does in fact occur.

The Metabolic Stability of DNA

If, as is widely supposed, DNA is an essential constituent of genetic material and of the chromosomes, it is clearly reasonable to expect some relationship between the chromosome complement of a cell and its DNA content. Apart from a few well-defined exceptions all cells of the body in a given species contain a constant number of well-defined chromosome pairs—the diploid number of chromosomes, while the sperm cells contain half this number of chromosomes—the haploid number. We might reasonably expect to find therefore that all the diploid cells in the tissues of any one species would contain the same amount of DNA but that the amount would vary from one species to another.

clear cut to make it evident that the DNA structure must allow for sufficient variation either in composition or configuration to account for the specific biological properties of certain molecular species.

Such differences in molecular composition have already been demonstrated in Table 2 from which it is clear that DNA's from different sources may have very different molar proportions of bases. Some even contain unusual bases such as 5-methyl cytosine which is absent from the DNA's of most micro-organisms but present in small amounts in mammalian DNA and plant DNA. An even more striking case is the DNA of certain strains of coliphage which contains hydroxymethyl-cytosine in place of cytosine.

But it is also possible for DNA's from the same or different sources to be distinguished from each other by differences in the sequence of bases along the polynucleotide chain even although the overall composition remains unchanged. It has been calculated for example that if the nucleic acid molecule contains 150 purines of two kinds and 150 pyrimidines of two kinds (and most DNA molecules contain more than this) the total possible number of isomers is about 4×10^{87}. This is an astronomical figure the magnitude of which is difficult to grasp but it does emphasize the fact that an enormous number of DNA molecules can be constructed on the same basic plan but with minor differences in the sequence of the different purines and pyrimidines along the chain. Consequently we might expect that the DNA in a cell would not be a single substance but a collection of very similar molecules differing slightly from each other in some respect. And if, as has been indicated above, DNA is intimately concerned in the question of genetic specificity, it would be logical to presuppose the existence of a large number of different molecular species, of different kinds of DNA molecules, in any one nucleus. Any attempt to separate all these different closely related DNA's occurring in the one type of cell nucleus would obviously be an exceedingly difficult, if not impossible, task, but some separation into groups has already been achieved. Brown and Watson (1953) in London and Chargaff and his colleagues in New York (Chargaff *et al.*, 1953;

chemical constituents we can calculate the amount of each constituent present in the average cell. This method of expressing tissue composition as amounts of a constituent per cell or per unit of DNA, which is the same thing, has much to commend it. It may for example be used in the comparison of the livers of male and female rats (Table 4). If the analytical results are expressed in the conventional way in terms of milligrams of a tissue constituent per hundred grams of tissue, differences between the sexes are not very striking nor are they statistically significant, but if tissue composition is expressed in terms of units of DNA in order to show the comparison on a per cell basis, a very different picture emerges and the differences obtained are highly significant.

For this type of work the liver is in many ways a peculiarly complicated tissue since in addition to polyploidy, it shows the presence of a good many binucleate cells. Harrison (1953) has devised a method of calculation whereby it is possible to allow not only for polyploidy but also for binucleate cells and for extracellular tissue in the calculation of the composition of the mean liver cell.

DNA IN CELL DIVISION

The helical structure of DNA (Figure 2) proposed by Watson and Crick provides a basis for accurate duplication at cell division, for the fact that one chain is the complement of the other with the order of bases reversed immediately suggests a mechanism by which the DNA molecule might duplicate itself at cell division. If the two chains could unwind and separate, each could then act as a mould or template on which a complementary chain might be built but it is difficult to understand how the two chains could untwist and come apart without forming a hopeless tangle, although a most ingenious solution to this problem has been proposed by Delbruck (1954).

Since the DNA content of the cell nucleus is characteristic of the species and since it varies within only very narrow limits, it is clear that in a rapidly growing tissue there must come a time in the course of the mitotic cycle when the DNA content of a dividing cell doubles itself. The exact stage at which this doubling occurs is still a matter of dispute but most evidence

The first experimental investigation of this question was made in 1948 by Boivin, Vendrely and Vendrely who isolated nuclei in bulk from tissues of different species, counted them and by estimating the DNA of the nuclei in bulk, calculated the mean amount of DNA per nucleus. This amount was found to vary from species to species but to be essentially the same in the different organs of any one species. Spermatozoa containing the haploid number of chromosomes contain approximately half the amount of DNA found in the corresponding diploid cells (Vendrely, 1955).

TABLE 4 Composition of rat liver tissue in terms of lipid phosphorus (LP), protein nitrogen (PN), ribonucleic acid phosphorus (RNA—P) and deoxyribonucleic acid phosphorus (DNA—P)

	mg./100g. tissue				pg./pg. DNA—P			
	LP	PN	RNA–P	DNA–P	LP	PN	RNA–P	Tissue *mass*
Male	133	2523	93	22	6 3	120	4 4	4710
Female	124	2522	100	27	4 5	92	3 6	3650
Pregnant female	135	2531	115	24	5 6	107	4 8	4180

1 pg (picogram) = 10^{-12}g

These results have been confirmed in several laboratories in different parts of the world both by the method of bulk chemical analysis of counted numbers of nuclei and by the measurement of the DNA content of large numbers of individual nuclei by *microspectrophotometric methods.* The latter methods give a more detailed picture showing a moderate scatter of individual values around the mean, so that although the DNA content of cell nuclei for any one tissue cannot be regarded as an absolute constant, the variations are small enough to justify the use of the mean figure for purposes of calculation

These observations have important implications (Davidson and Leslie, 1950a, b); for example, (i) if we know the amount of DNA per nucleus for a given tissue and if we estimate chemically the total amount of DNA in a sample of that tissue, we can easily calculate the number of nuclei and hence the number of cells present; this calculation is of course of great value in studies on cell growth; and (ii) if we analyse the tissue for its other

shows a very low uptake of the isotope. Indeed in such a non-proliferating tissue DNA is probably the most metabolically inert substance in the cell. On the other hand in tissues like bone marrow in which mitotic activity is pronounced, the DNA takes up labelled precursors very rapidly. The lymphoid tissue of appendix likewise shows a high activity while such tissues as intestinal mucosa or thymus give intermediate values.

The differences in the behaviour of the DNA's in various tissues are shown up even more clearly in the curves in Figure 3 where the incorporation is plotted against time. One effect of X-rays and mitotic inhibiting substances is to inhibit incorporation into DNA without greatly affecting that into RNA.

DNA in Bacteriophage

Some of the most interesting recent developments in relation to DNA have been made on the various bacteriophages which attack *E. coli*. The coliphages of the T series (T_2, T_4 and T_6) are submicroscopic particles, tadpole-like in structure, consisting of about 60 per cent protein and 40 per cent DNA. In the T-even coliphages the DNA is unusual in containing the base hydroxymethyl cytosine in place of cytosine (Table 2).

When bacterial cells are infected with coliphage the phage particles are adsorbed on the bacterial surface and multiply inside the bacterial cell, ultimately causing its lysis with liberation of a large number of new phage particles.

In some very beautiful experiments Hershey and Chase (1952) have clarified the nature of this process by labelling the DNA *of the* T_2 *and* T_6 *phage with* ^{32}P *and the phage protein* with ^{35}S. Their results have shown clearly that while the phage DNA enters the bacterial cell the phage protein does not. Indeed the phage particle appears to adhere to the bacterial surface by means of its tail and to act as a microsyringe injecting its DNA complement into the cell. The protein residues of the phage particle remain outside and can even be stripped off the bacterial surfaces leaving only a small part of the protein *attached to the infected cell.* When the stripped cells are lysed, even this small residue of phage protein can all be recovered in the bacterial *débris*. Thus it appears that phage DNA alone is

appears to be in favour of interphase or just before prophase (Vendrely, 1955). It would be reasonable therefore to expect that the DNA in dividing cells would show a particularly high metabolic activity which would be absent in a non-growing

TABLE 5. Incorporation of labelled precursors into whole DNA or DNA adenine in rabbit tissues in a 2-hr. period. The figures are given relative to bone marrow as 100. (Smellie and Davidson, 1955)

Precursor	^{32}P (whole DNA)	^{14}C—Formate (DNA adenine)	[8-^{14}C]-Adenine (DNA adenine)
Tissue			
Bone marrow	100	100	100
Appendix	*106*	*61*	*70*
Intestinal mucosa	20	26	32
Kidney	6	1	—
Liver	5	3	7
Spleen	12	8	40
Thymus	33	27	32

tissue, but which could be demonstrated by determining the rate of incorporation of isotopically labelled precursors into the DNA molecule. Whatever the precursor the pattern which emerges is essentially the same (Table 5). As one might expect, the DNA of non-proliferating tissues such as liver or kidney

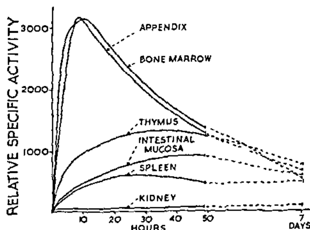


FIG. 3. Incorporation of ^{32}P into the DNA of several rabbit tissues at different time intervals after administration of the isotope (Smellie *et al.*, 1955)

citric nuclei (CN) are excellent for many purposes they suffer from the defect that all the nuclear constituents soluble in dilute citric acid are lost in the course of preparation. This defect may be overcome by using the technique originally devised by Behrens and subsequently modified by other workers (Dounce *et al.*, 1950; Allfrey *et al.*, 1952; Kay *et al.*, 1956). In this method the tissue is dried from the frozen state, ground to a fine powder and suspended in a series of organic solvents of varying densities so as to separate the nuclei from the cytoplasmic *débris*. The process is tedious and technically difficult but it results in the production of satisfactory specimens of clean nuclei with all the water-soluble constituents (though not the lipids) intact. Such non-aqueous nuclei (NAN) are illustrated in Plate I, Figure 5*b*.

The chief chemical constituents of cell nuclei are of course proteins and nucleic acids. The protein components include both histones and non-histone proteins of more than one type, while the nucleic acids include all the DNA of the cell and a small but important proportion of its RNA. Some of this RNA is located in the nucleolus but recent experiments with isolated nucleoli have shown that the nucleolar RNA varies enormously in amount from one cell to another and in no cases accounts for all the nuclear RNA. The remainder is in the chromosomes along with most of the DNA.

The RNA of the cell nucleus differs from that of the cytoplasm both in chemical structure as revealed by the molar proportions of bases and in its metabolic activity (Crosbie *et al.*, 1953; Smellie *et al.*, 1955). It incorporates labelled precursors much more rapidly than does cytoplasmic RNA (Figure 4 *a* and *b*) in all tissues which have so far been examined and this distinction is evident whether ^{32}P, ^{14}C-formate or [8-^{14}C]-adenine is used as a precursor, although it is more pronounced with ^{32}P. The function of this nuclear RNA is still unknown, but experiments with non-aqueous nuclei (NAN) have shown that such nuclei do in fact contain two types of RNA, one type with a rapid turnover which is also found in citric nuclei and a second type with a much slower turnover which can be extracted from NAN with citric acid, together with 10–25 per cent of the

able to bring about the process of infection, the phage protein having acted merely as a protective covering.

When the bacterial cells are infected with phage DNA their metabolism is diverted exclusively to phage production. Respiration remains constant instead of increasing as would happen in a normal growing culture. The enzymes and RNA of the host no longer increase. On the other hand synthesis of phage DNA and protein goes on actively. The phage DNA therefore acts as a parasite on the nucleic acid and protein metabolism of the host so that new phage DNA of its specific and peculiar type is built up first at the expense of the host DNA and then from small molecular precursors. Here, then, is an excellent example of the power of DNA to initiate its own reduplication and the formation of its associated specific protein.

The Distribution of Ribonucleic Acid

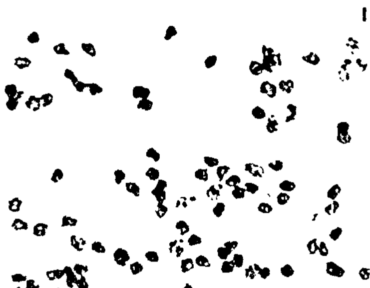
The investigation of the ribonucleic acid of the cells is complicated by the fact that RNA is located in more than one cell constituent and its study therefore involves either an examination of the different parts of intact cells by such techniques as autoradiography or ultramicrospectrophotometry, or the disruption of the cells with separation of the morphological fractions for bulk chemical examination. When mammalian cells are broken up in a sucrose medium and centrifuged, the various constituents can be brought down in stages by the application of increasing centrifugal force. Nuclear material comes out most readily, then the mitochondria. Higher speeds bring down the submicroscopic particles known as *microsomes* which according to one school of thought are derived from the endoplasmic reticulum (Porter, 1954) and which form the chromophilic ground substance of the cell. The non-sedimentable material is considered to be derived from the cell sap. Each of these cell fractions contains RNA.

The nuclear material separated from a sucrose dispersion of cellular material is heavily contaminated with cytoplasmic *débris* but excellent clean preparations of nuclei can be obtained from dispersions in citric acid (Plate I, Figure 5*a*).[1] While such

[1] This plate will be found facing page 48.



a



b

FIG 5 *a* Rabbit thymus nuclei isolated by the citric acid procedure. *b* Rabbit thymus nuclei isolated from non-aqueous solvents. (Magnification × 730) (Preparations by Dr. E R M Kay)

nuclear protein. Cell nuclei prepared by the conventional citric acid method can therefore tell us only part of the story of nuclear metabolism.

Only a small amount (in liver about 10 per cent) of the RNA of the cell is located in the nucleus, the remainder being found in the cytoplasmic fractions, mitochondria, microsomes and cell sap. The RNA's in these three cell fractions are of similar

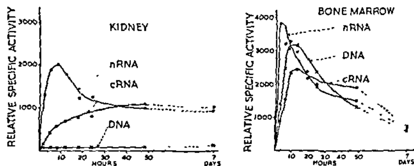


Fig. 4. Incorporation of ^{32}P into the DNA, nuclear RNA (nRNA), and cytoplasmic RNA (cRNA) of (*a*) kidney and (*b*) bone marrow of rabbits at different time intervals after administration of the isotope.

chemical composition as regards molar proportions of bases but differ slightly in their patterns of uptake of labelled precursors (Smellie *et al.*, 1953; Moldave and Heidelberger, 1954). RNA is least abundant in the mitochondria in which its function is quite unknown, although the mitochondria themselves appear to act as what has been described as the 'power plant of the cell' since they contain the enzymes required for oxidative phosphorylation (Hogeboom, Schneider and Striebich, 1953).

RNA and Protein Synthesis

The microsomes contain abundant RNA and are generally considered to be concerned in some as yet rather obscure way with the process of protein synthesis. No attempt will be made to deal with the subject here in detail, but it will be appropriate at this stage to make a few critical comments on the supposed connection between RNA and the synthesis of proteins.

The early observations summarized by Caspersson (1950) and by Brachet (1952) working independently and with different methods of approach, the former using ultraviolet microspectrophotometry and the latter using histochemical procedures, revealed that RNA was particularly abundant in cells engaged in the synthesis of proteins either for growth or for secretion, e.g. in embryonic tissues, in tumours, in rapidly growing bacterial cultures, in the exocrine cells of the pancreas and so on. All these cells are characterized by large nucleoli.

The application of quantitative chemical methods has made it clear that synthesis of RNA always precedes protein synthesis in fibroblast cultures, and that in bacterial cultures the RNA content is proportional to the growth rate. Indeed in the logarithmic phase of growth the synthesis of RNA runs parallel with that of protein, and adaptive enzyme formation is accompanied by a simultaneous rise in RNA content.

It is, however, possible to dissociate the two processes. In certain bacteria, for example, the application of antibiotics such as chloramphenicol, or of cobaltous ions, will inhibit protein synthesis but not RNA synthesis.

At this point it is perhaps desirable to consider briefly the different possible criteria of protein synthesis. In dealing with relatively simple systems such as bacterial cultures, protein synthesis may be measured by the growth in numbers of the organisms or by the increase in protein nitrogen, but such methods cannot readily be applied to mammalian tissues, except, within limits, to tissue cultures. A more precise and specific method is to estimate the amount of some specific protein produced under controlled conditions. Such a protein might be an enzyme such as amylase produced by pharmacological stimulation of sections of glandular tissue (e.g. pancreas) or it might be an adaptive enzyme produced in a bacterial culture by exhibition of the appropriate substrate. Another criterion commonly employed is to measure the rate of incorporation of labelled amino-acids into cellular proteins but this method is not altogether free from objection since it is not impossible to imagine that the incorporation of an amino-acid at any particular point in a protein molecule may be the result

formation of new enzyme molecules, and by Lester (1953) who showed that *Micrococcus lysodeikticus*, after lysis with lysozyme, was able to incorporate amino-acids into its proteins in the absence but not in the presence of ribonuclease. This effect was confirmed by Beljanski (1954) who also showed that the effect of ribonuclease was not on the oxidative mechanisms of the cell.

In preparations from animal cells ribonuclease inhibits incorporation of amino-acids into the proteins (Allfrey *et al.*, 1953) while in living respiring cells (onion roots) ribonuclease likewise inhibits incorporation of labelled amino-acids without affecting respiration (Brachet, 1954). Moreover the observation that adaptive enzyme formation by bacteria is inhibited by ultraviolet light and that the degree of inhibition at different wavelengths runs parallel with the ultraviolet absorption by the nucleic acids, is also good evidence for the participation of the nucleic acids in protein synthesis (Swanson, 1950; Jacob *et al.*, 1951).

All this evidence is impressive but it is not without its critics. Cavanna *et al.* (1955) for example found no correlation in *E. coli* between the amounts of RNA, DNA and protein per cell and the rate of cell fission, while Hokin and Hokin (1954) found that the stimulation of enzyme secretion by pancreatic tissue was not accompanied by an increased rate of RNA synthesis or turnover.

Moreover despite all the evidence of correlation in time and place between RNA and protein synthesis, no satisfactory theory has yet been put forward to explain the mechanism by which RNA acts either in an energy-supplying capacity or as a template for the alignment either of amino-acids or of peptidases in patterns (Hokin and Hokin, 1954). The detailed and ingenious suggestion of Dounce (1952) who envisages a phosphorylated RNA is still highly speculative.

The verdict for the participation of RNA in the process of protein synthesis must therefore still remain as 'not proven'.

RNA and Steroid Metabolism

In conclusion it may be of interest to record some preliminary experiments carried out in collaboration with Professor T.

of an enzymically controlled exchange taking place without *either rupture of the rest of the molecule or the formation of* new molecules. Nevertheless the application of this technique has focused attention on the microsomes as probable organelles for protein synthesis.

It has for instance been shown by many workers that labelled amino-acids are incorporated more readily into the proteins of the microsomes than into those of any other cell fraction. The microsomes moreover are not only exceedingly rich in RNA but their content of RNA bears a close relationship to the rate of protein synthesis in the tissue from which they are obtained (Allfrey *et al.*, 1953). Siekevitz (1952) has gone a stage further in showing that incorporation of labelled alanine into the microsomes in liver tissue is dependent on the presence of mitochondria which are the seat of oxidative phosphorylation and which apparently produce a factor, perhaps allied to ATP, which promotes incorporation of amino-acids into microsomal protein, while Zamecnik and Keller (1954) have confirmed that such incorporation into the proteins of isolated microsomes is an anaerobic process requiring only an ATP generating system and a non-dialysable soluble cell fraction provided that *the RNA of the microsomes is intact.*

Since no separate microsome fraction can be obtained from bacterial cells, in experiments with bacteria the RNA cannot be morphologically subdivided but bacterial cells can provide useful information. Gale and Folkes (1954), using disintegrated staphylococcal cells which no longer respired, found that labelled amino-acids could still be incorporated into the proteins *in the presence of hexosediphosphate and* ATP. When such preparations were treated with nucleases or extracted with molar sodium chloride to remove nucleic acids, incorporation was reduced but was restored by addition of nucleic acids. Such staphylococcal preparations could also bring about the synthesis of catalase, or the adaptive synthesis of β-galactosidase in the presence of nucleic acids, but although bacterial RNA was most effective *it was not specific. Confirmation of* these results has been obtained by Spiegelman (1954) who concluded that synthesis of new RNA is a compulsory concomitant for the

DELBRÜCK, M. (1954). *Proc. nat. Acad. Sci. U.S A.* **40**, 783.
DOUNCE, A. L., TISHKOFF, G H., BENNETT, S. R and FREER, R. M. (1950) *J. gen. Physiol.* **33**, 629.
DOUNCE, A. L. (1952). *Enzymologia*, **15**, 251.
FRANKLIN, R. E. and GOSLING, R. G. (1953). *Nature*, **172**, 156.
GALE, E. F. and FOLKES, J P. (1954). *Nature*, **173**, 1223
HARRISON, M (1953). *Proc. roy. Soc.* B, **141**, 203.
HECHTER, O. (1953). Ciba Foundation Colloquium on Endocrinology, vol. 7, p 172.
HERSHEY, A. D and CHASE, M. (1952). *J. gen. Physiol.* **36**, 39
HOGEBOOM, G. H., SCHNEIDER, W. C and STRIEBICH, M J. (1953). *Cancer Res.* **13**, 617.
HOKIN, L E. and HOKIN, M. R. (1954). *Biochim biophys. Acta*, **13**, 401.
HOTCHKISS, R. D (1955). In *The Nucleic Acids—Chemistry and Biology*, ed E. Chargaff and J. N. Davidson, vol. II, p. 435.
JACOB, F., TORRIANI, A. M. and MONOD, J. (1951). *Comp. rend. (Paris)*, **233**, 1230.
KAY, E. R. M., SMELLIE, R. M. S., HUMPHREY, G. H. and DAVIDSON, J. N [illegible]
[illegible] 6, 679
[illegible] N. and DAWSON, I M. (1953). *Biochem. J* **54**, 280
SMELLIE, R. M. S. and DAVIDSON, J. N. (1955). Unpublished results.
SMELLIE, R. M S, HUMPHREY, G. H, KAY, E. R. M. and DAVIDSON, J. N (1955). *Biochem. J* **60**, 177.
SPIEGELMAN, S (1954). Eighth International Congress for Cell Biology (Proceedings)
SWANSON, P. A. (1950). *Proc. nat. Acad. Sci., U.S A.* **36**, 699.
[illegible]
Nature, **172**, 759
ZAMECNIK, P. C. and KELLER, E B (1954). *J biol Chem* **209**, 337

Symington on the nucleic acids in the adrenal gland. The RNA phosphorus content of human adrenal tissue varies from 15 to 28 mg. per 100 g. As the result of stimulation with ACTH it rises sharply to 40 to 50 mg. per 100 g. while the DNA concentration remains unaltered. A similar rise is found in the glands of patients dying under conditions of stress. These rises are very beautifully illustrated in sections of adrenal tissue stained to demonstrate RNA and protein. The significance of these observations is obscure but it may well be, as Hechter (*1953) has suggested, that ACTH stimulates the synthesis of the* proteins involved in corticosteroidogenesis. If so, this is yet another example merely of the association of RNA and protein synthesis (Symington and Davidson, 1956).

REFERENCES

ALLFREY, V., STERN, H., MIRSKY, E. A. and SAETREN, H. (1952) *J. gen Physiol* 35, 529

ALLFREY, V , DALY, M. M. and MIRSKY, A. E. (1953). *J. gen. Physiol.* 37, 157.

AVERY, O T , MACLEOD, C. M and MCCARTY, M. (1944). *J. exp. Med.* 79, 137.

BELJANSKI, M (1954). *Biochim. biophys Acta*, 15, 425.

BOIVIN, A., VENDRELY, R. and VENDRELY, C. (1948). *C R. Acad. Sci. Paris*, 226, 1061

BRACHET, J. (1952). *Actualités biochimiques No 16.* Paris, Masson

BRACHET, J. (1954) *Nature*, 174, 876.

[illegible] N. A, [illegible]

CRAMPTON, C. F., LIPSHITZ, R and CHARGAFF, E. (1954) *J. biol Chem* 211, 125.

CROSBIE, G. W., SMELLIE, R M. S. and DAVIDSON, J N. (1953) *Biochem J.* 54, 287.

DAVIDSON, J. N and LESLIE, I (1950a). *Nature*, 165, 49.

DAVIDSON, J N and LESLIE, I (1950b) *Cancer Res.* 10, 587.

the right spot and to the right degree. Abnormalities of growth in the widest sense may develop if this normal pattern of integrated activities is upset (cf. Bergel, 1954a). This statement, which may sound rather superficial, touches on a possible mechanism of their causation. But before mentioning the problem of causative agents, and thus induction of abnormalities, one should consider Table 1, which gives a list of histo- and cyto-morphological groups. Leaving aside the forms of reductive growth-abnormalities, hypoplasia and atrophy, and starting from normal growth events, the expert will recognize on a cellular, tissue or whole body level, abnormalities of organs, muscles, blood vessels, glands, skin, etc., showing the phenomena of hypertrophy or hyperplasia or both together. Apart from an over-all enlargement, e.g. uterus and breast during pregnancy, muscles in the strong man, kidney after loss or degeneration of the other, representing cases of physiological, adaptive and compensatory hypertrophy respectively, irregularities can develop which disturb in most cases only locally, the species-conditioned design of the body. Such out-growths can take the form of swellings, warts, polypi, papillomas, palpable tumours. As long as they are still under the control of the whole organism they represent benign types of abnormalities which only in rare cases become a threat to life. Most pathologists do [illegible]

to develop anaplasia, invades normal tissue, gives cause to the formation of secondaries, abnormally stimulates blood supply, loses with its cells mutual adhesiveness, and on an experimental basis becomes transplantable, and is easily maintained in the form of tissue cultures, then one is dealing with one or the other form of cancer. Summarily, it should be stated that the term 'abnormal growth', as far as it is used in this paper, will include the wider meaning of growth as discussed and will refer to all morphological groups just mentioned, that is, hypertrophic, hyperplastic and neoplastic states. It should be added that no distinction will be made between *spontaneous* and *experimentally* produced tumours.

IV

Some Chemical Aspects of Abnormal Growth

F. BERGEL

INTRODUCTION

THE term 'growth' can be used in a much wider sense than its didactic and pedantic meaning in biology would allow (cf. Bergel, 1953). There, in respect of animals and man, it signifies increase, at certain rates, in size of different tissues, organs and the body as a whole, due to the enlargement and division of various groups of cells But there are a number of phenomena which are so closely connected and often concomitant with growth proper that one cannot separate them completely from it without introducing artificialities. They are at the time of embryonic growth, the processes of differentiation and morphogenesis, carrying with them the elements of qualitative changes and form; during the life of the individual they are tissue maintenance and repair, reproduction, and even the controlled arrest of growth in those parts of the organism which

going by intake of food, water and oxygen and comprising all reactions which provide various forms of energy and which take care of the production and disposal of cellular substances; whereby the former, the syntheses, consist to a large extent of exact reduplication processes of many specific constituents. This

General Chemical Aspects

This takes one to the subject proper—'Chemical Aspects' (cf. Bergel, 1954b). It would be more honest to call them chemical problems as is done on Table 2. What are they? Roughly speaking they can be divided into three main groups, always referring to biochemical, organic-inorganic and physico-chemical approaches.

Table 2. Chemical* Problems

I. Agents causing Abnormal and Malignant Growth (Carcinogenic agents)
- Their isolation and synthesis,
- their physico-chemical and metabolic properties,
- their mode of action,
- their interaction with cellular constituents,
- *their reversible and permanent effects.*
- The problem of endogenous agents;
- small and macromolecular structures:
- hormones, growth regulators, viruses and cellular particles (genetic material).

II. Chemistry of Abnormalities in Comparison with Normal, Regenerating and Embryonic Tissue Chemistry* of Tumour-Bearing Host*
- Comparative Chemistry* of cellular constituents:
- *nucleic acids, proteins, etc;*
- enzyme and coenzyme pattern;
- trace element concentrations;
- energy metabolism, anabolic and catabolic pathways
- Chemical* cause of rapid division, cytological irregularities, invasiveness, reduced mutual adhesiveness, dedifferentiation and autonomy, stimulation of blood supply, metastases formation, etc.

III. Chemotherapy of Abnormal Growth and Cancer (including reticuloses)
- Cytotoxic Agents (alkylating agents)
- Difference between anti-tumour and carcinogenic effects.
- Antagonists.
- Hormones and anti-hormones.
- Biological material for restitutional therapy.
- Radioactive Isotopes (chemical effects of radiations)
- Products from bacteria, moulds and plants.
- Problem of drug resistance

* Chemistry stands for Biochemistry, Organic and Inorganic and Physical Chemistry

Group 1 includes the chemistry of agents causing abnormal growth. One will find in the Table most of the sub-problems of

TABLE 1. Histo- and Cyto-Morphological Groups

		Metaplasia	Embryonic	G		
				R	Hypoplasia	
Neoplasia——Hyperplasia			Normal	O		
						——Aplasia
malignant——benign		Hypertrophy (Physiological, Adaptive, Compensatory)	Regenerative	W		
anaplastic autonomous	differentiated dependent		Repair and Maintenance	T	Atrophy (Old Age, Disuse, Starvation)	
				H		

TABLE 3. Some Primary Causative Agents and Factors of Abnormal Growth and Development
(Carcinogenic ones are shown in italic)

MECHANICAL
- Injury and Wounds
- Burns
- Loss of one half of a double organ
- Excessive use, Disuse
- Pressure
- Friction
- Irritation.

PHYSICAL
- Heat, *Cold*
- *Ionizing Radiations (U.V., X-rays, β-rays)*
- *Polymers (Cellophane and other polymers).*

CHEMICAL AND BIOCHEMICAL
- Drugs, antibiotics
- *Hormone imbalance* (Sex hormones, corticosteroids, pituitary and thyroid hormones)
- Vitamin deficiencies
- Change of *coenzyme* and *enzyme pattern*
- Starvation
- Lack or excess of oxygen
- *Carcinogenic Substances*·
- Inorganic (Ni, Co, As, Be, Zn)
- Organic: Aromatic polycyclic hydrocarbons,
- heterocyclic compounds,
- aromatic amines, azo-derivatives and amino-stilbenes,
- monofunctional alkylating agents,
- anti-tumour agents, such as aliphatic and aromatic nitrogen mustards, diepoxides, dimesyloxy derivatives, methylolamides,
- urethane,
- xanthine.

PHYSIOLOGICAL
- Pregnancy
- Exercise

GENETICAL
- Genes, carrying abnormal characters
- Loss of genes causing abnormal cellular behaviour and change of metabolic and constitutional pattern

BIOLOGICAL
- Parasites
- Micro-organisms
- *Viruses*
- *Cellular particles.*

this aetiological group including, for instance, the mode of their action and the problem of endogenous agents. Group 2 deals with the chemistry of abnormal in comparison with normal tissue and with the chemistry of the tumour-bearing host. It embraces also the study of the chemical causes of all those phenomena characteristic of malignancy as enumerated before. All this is of the greatest importance for finding a weak point, to be attacked, selectively if possible, by drugs of Group 3, which comprises all attempts at chemotherapy of any form of abnormal growth as defined, but particularly of cancer and reticuloses. It is obvious that the items under these group headings, and no completeness is claimed, are so numerous that it would require a whole series of lectures to cover even part of the field with its many unsolved riddles. It is therefore proposed to touch only lightly on items of Group 3. Boyland (1954) has spoken on this subject in this series of lectures and on 9 December 1954 Haddow dealt with one important class, the nitrogen mustards. This leaves on the whole, Groups 1 and 2. In order to give some over-all impressions not only of the subject but also of the great difficulties inherent in it, it is necessary to make an arbitrary choice from the many problems presented by them.

Problems connected with the Causation of Abnormal Growth

In Table 3 an attempt is made to assemble some of the primary causative agents and factors of abnormal growth and development. Leaving aside those involved in abnormal development, a whole story of its own, and physiological ones, like pregnancy and exercise, one is always struck by the diversity of the remainder and consequently by the probable diversity of their modes of action. In the case of benign abnormalities the cessation of action of the disturbing factor leads often to arrest and regression of the outgrowth, the tumour is still dependent on the 'irritant' and is not autonomous But the big mystery of true carcinogenesis lies in the fact that even a long time after the action of the agent has ceased, the abnormality continues and has become self-perpetuating. How ever this happens, it is necessary to assume that something in the cell has permanently

fractions of tumour cells, allegedly free of intact cells or nuclei, by differential centrifugation. Particularly their nuclear chromatin fraction from a rat lymphosarcoma produced on subcutaneous injections a considerable number of identical tumours, and an even higher incidence of leukaemia (see Table 4). Klein (1952) in Sweden has confirmed this observation in mice but he is highly critical of the significance of these experiments as, working with pure and hybrid strains of mice he obtained on transfer lymphosarcomas showing the genetic characteristics of

TABLE 4. The Incidence of Lymphosarcoma and Leukaemia following the Subcutaneous Injections of Tumour Cells and Isolated Cell Particles. From Stasney *et al* (1952)

Material	Total	Tumour	Leukaemia
Intact cells	262	202	25 (12%)
Chromatin	134	44 (33%)	19 (43%)
Mitochondria	58	3 (5%)	1 (33%)
Chromatin and mitochondria	9	4 (44%)	2 (50%)

the donor and not the host. This may be due to the fact that the complex chromatin material carries other somatogenetic properties apart from carcinogenic potentialities. If this could be established beyond doubt then one could assume that mammalian nuclear material or perhaps nucleo-proteins act *vis-à-vis* normal animal cells as the known transforming factors, identified with specific deoxyribonucleic acids, behave towards bacteria, such as *pneumococci*, *H. influenzae*.

Whilst this is an example of the possible participation of nuclear material in carcinogenesis, what about the causation of benign forms of hyperplasia and hypertrophies, from which malignancy may develop and whose mode of formation could assist in the understanding, from a chemical point of view, of that of neoplasms? One recent report comes from the Max Planck Institute for Biochemistry in Germany, where Friedrich-Freksa and Zaki (1954) have found that regenerating liver of partially hepatectomized rats released non-dialysable, thus probably high-molecular, substances into the blood stream. When injected in the form of serum into normal rats the near-resting liver, but not other tissue such as parotis, showed a

changed, possibly in the apparatus safeguarding exact reduplications of vital constituents. Everybody and particularly the biologist and chemist engaged in cancer research is most curious, as to where, and molecularly speaking, with what this change has taken place: In the chromosomal material of the nucleus, in the plasmagenes or other particles of the cytoplasm and thus representing a somatic mutation? Or maybe, to follow Hinshelwood's (1952) studies of adaptation phenomena in bacteria, in the relative concentrations of enzymes and substrates forming in a steady state a system of interlocking parts and following the exponential law of autocatalysis? Although one cannot answer yet these questions with certainty, a number of experimental results will be mentioned which may throw at least some light on this vexed problem. Nearly twenty years ago, Haddow (1935) established that carcinogenic agents also show anti-tumour effects. Vice-versa a great number of compounds, prepared as anti-tumour drugs during recent years, have been found to be carcinogenic, including bi- and poly-functional alkylating agents, i.e nitrogen mustards, ethyleneimines, etc. It was only recently that Walpole *et al.* (1954) published their results with certain monofunctional derivatives of ethyleneimine which produce only carcinogenic but hardly anti-tumour and chromosome damaging effects. This is very interesting, because it establishes a case for near-separation of carcinogenicity from tumour-destructive activity. Whilst the differences appear to be not truly qualitative but still quantitative ones, they invite the speculation that the cellular loci or components involved in carcinogenesis may not be identical with those concerned with tumour inhibition. Whether such components are nuclear or cytoplasmic or both, whether they are in one instance proteins, in the other nucleic acids, one does not know. It would be too much to expect at this stage a reconciliation between the somewhat opposing hypotheses for carcinogenesis and the development of the typical characteristics of malignancy, namely of somatic mutation, viruses and alterations in Hinshelwood's coordinated enzyme reactions. The possibility of purely nuclear changes prior to or during malignancy seems to be strengthened by an observation of Stasney *et al.* (1950, 1952) who prepared

fractions of tumour cells, allegedly free of intact cells or nuclei, by differential centrifugation. Particularly their nuclear chromatin fraction from a rat lymphosarcoma produced on subcutaneous injections a considerable number of identical tumours, and an even higher incidence of leukaemia (see Table 4). Klein (1952) in Sweden has confirmed this observation in mice but he is highly critical of the significance of these experiments as, working with pure and hybrid strains of mice he obtained on transfer lymphosarcomas showing the genetic characteristics of

TABLE 4. The Incidence of Lymphosarcoma and Leukaemia following the Subcutaneous Injections of Tumour Cells and Isolated Cell Particles. From Stasney *et al* (1952)

Material	Total	Tumour	Leukaemia
Intact cells	262	202	25 (12%)
Chromatin	134	44 (33%)	19 (43%)
Mitochondria	58	3 (5%)	1 (33%)
Chromatin and mitochondria	9	4 (44%)	2 (50%)

the donor and not the host. This may be due to the fact that the complex chromatin material carries other somatogenetic properties apart from carcinogenic potentialities. If this could be established beyond doubt then one could assume that mammalian nuclear material or perhaps nucleo-proteins act *vis-à-vis* normal animal cells as the known transforming factors, identified with specific deoxyribonucleic acids, behave towards bacteria, such as *pneumococci*, *H influenzae*.

Whilst this is an example of the possible participation of nuclear material in carcinogenesis, what about the causation of benign forms of hyperplasia and hypertrophies, from which malignancy may develop and whose mode of formation could assist in the understanding, from a chemical point of view, of that of neoplasms? One recent report comes from the Max P[illegible] drich-F[illegible] ver of F[illegible] thus probably high-molecular, substances into the blood stream. When injected in the form of serum into normal rats the near-resting liver, but not other tissue such as parotis, showed a

changed, possibly in the apparatus safeguarding exact reduplications of vital constituents. Everybody and particularly the biologist and chemist engaged in cancer research is most curious, as to where, and molecularly speaking, with what this change has taken place: In the chromosomal material of the nucleus, in the plasmagenes or other particles of the cytoplasm and thus representing a somatic mutation? Or maybe, to follow Hinshelwood's (1952) studies of adaptation phenomena in bacteria, in the relative concentrations of enzymes and substrates forming in a steady state a system of interlocking parts and following the exponential law of autocatalysis? Although one cannot answer yet these questions with certainty, a number of experimental results will be mentioned which may throw at least some light on this vexed problem. Nearly twenty years ago, Haddow (1935) established that carcinogenic agents also show anti-tumour effects. Vice-versa a great number of compounds, prepared as anti-tumour drugs during recent years, have been found to be carcinogenic, including bi- and poly-functional alkylating agents, i.e. nitrogen mustards, ethyleneimines, etc. It was only recently that Walpole *et al.* (1954) published their results with certain monofunctional derivatives of ethyleneimine which produce only carcinogenic but hardly anti-tumour and chromosome damaging effects. This is very interesting, because it establishes a case for near-separation of carcinogenicity from tumour-destructive activity. Whilst the differences appear to be not truly qualitative but still quantitative ones, they invite the speculation that the cellular loci or components involved in carcinogenesis may not be identical with those concerned with tumour inhibition. Whether such components are nuclear or cytoplasmic or both, whether they are in one instance proteins, in the other nucleic acids, one does not know. It would be too much to expect at this stage a reconciliation between the somewhat opposing hypotheses for carcinogenesis and the development of the typical characteristics of malignancy, namely of somatic mutation, viruses and alterations in Hinshelwood's coordinated enzyme reactions. The possibility of purely nuclear changes prior to or during malignancy seems to be strengthened by an observation of Stasney *et al.* (1950, 1952) who prepared

rodents kidney hypertrophy due to stimulation of mitotic activity in the tubular epithelium. This abnormal state is transient, as can be seen from Figure 1. There the kidney weights divided by body weight are compared with normal controls. In addition to xanthopterin which might be accused of producing sometimes the hypertrophy by irritation effects, due to its deposition as micro-crystals in the tubules, other pteridines, pteridine analogues, purine analogues and a miscellaneous group of substances, which in part have been synthesized by

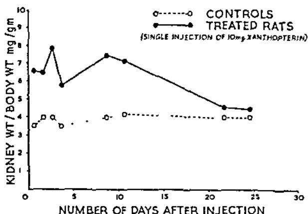


FIG 1

Timmis *et al.* and Ross, have been tested by Haddow and Horning (1951, 1953). A number which did not show this deposition of crystals in the tubules have been found active (see Table 5). Xanthopterin is known to act as substrate and in larger concentrations as inhibitor of the enzyme xanthine oxidase. Taking this as a starting point, Albert and Timmis (see Haddow *et al.*, 1951) independently put forward a hypothesis for the mode of action of xanthopterin and other substances causing kidney hypertrophy Following a substrate- or competitive inhibition of xanthine oxidase, either a metabolic accumulation of xanthopterin itself would promote cell division or in the case of xanthine accumulating this would reflect back on other purines, nucleotides and finally nucleic acids and thus,

maybe, on growth. There is no doubt about the vital rôle of xanthine oxidase as an enzyme controlling the last stages of nucleic acid catabolism by removal of hypoxanthine and xanthine in form of uric acid from the general pool of purines. In consequence, Bray and Avis (see Bergel *et al.*, 1952) measured the *in vitro* inhibition of bovine[1] xanthine oxidase by the substances tested for kidney hypertrophy; their results are given in Table 5. True, the correlation between the two effects does not always exist. Maybe the *in vivo* conditions or the bovine and rodent xanthine oxidases are different or other enzymes controlling nucleic acid metabolism, so far not tried, are involved. This is supported by two observations, one by Richert *et al.* (1950) who found that antabuse inhibited rat-liver xanthine oxidase but not the enzyme from cows' milk, and the other by Dietrich and Shapiro (1953) who reported that flavotin, a riboflavin analogue, antagonized directly the xanthine oxidase of a mouse mammary carcinoma (755) and indirectly, through substrate inhibition, guanase but not the corresponding enzymes from mouse liver *in vitro*. There are a number of other findings, also suggestive of an important part played by xanthine oxidase in the processes of normal and abnormal growth. Professor Haddow has kindly allowed me to mention, first (Haddow *et al.*, 1953), that in preliminary experiments spontaneous mammary tumours in mice responded with arrest of growth to relatively large doses of xanthine oxidase in form of concentrates from cows' milk and secondly that xanthine, one of its normal substrates, on long-term injection into rats has given rise to granuloma which on transplantation developed into sarcomas. The result with the mammary-tumour-bearing mice, somewhat related to recent experiments by Ledoux (1954) with another enzyme, ribonuclease, on ascites tumours, supports the concept of xanthine oxidase acting as a physiological purine-antagonist. On the other hand the induction of tumours by a normally occurring purine seems to be a further confirmation of the rôle which relative concentrations of substrates and enzymes may play in growth processes. That shorter administration of xanthine appears to increase the levels of xanthine oxidase and

[1] Wherever 'bovine' is used in this connection, it refers to cow's milk

related enzymes in mouse liver has been claimed by Feigelson *et al.* (1954). Their findings are rather reminiscent of adaptation phenomena in micro-organisms but as the enzymes involved already exist in the mammalian tissue it would be better to talk of modulation.

It can be imagined how interested the team at the Chester Beatty Research Institute is in xanthine oxidase itself, so far from bovine sources. Its composition is given at the bottom of Table 5. In the course of the work this rather complex metalloflavoprotein was obtained for the first time in a crystalline form (Avis *et al.*, 1954).

Taking all these observations together, it remains to be seen at present whether inhibition or stimulation, excess or deficiency of xanthine oxidase or metabolically related enzymes are behind the tissue changes just mentioned. At the same time the author and his colleagues are also anxious to establish the identity, or otherwise, of the enzyme in various species and organs and to widen the knowledge of its concentration in different kinds of tissues under normal and abnormal conditions.

Enzyme Pattern

This leads to, what was mentioned before, the comparative chemistry of cellular abnormalities or better still, the chemopathology of growth. Of course, xanthine oxidase is not the only flower in the field, and enzymes are not the only group of cell constituents which has received the chemist's attention. Looking at Table 6, redrawn from Winzler's table in *Physiopathology of Cancer* (1953), on the left are the main groups of substances and any gross changes on the right. So far, no specific chemical has been found which is either solely present in, or totally absent from, abnormal growth as compared with embryonic, regenerating or adult tissues. The differences which may vary from tumour to tumour are only of a quantitative nature. One does not wish to exaggerate the technical difficulties which the chemists encounter in this kind of work but there are many, starting with the selection of homologous tissues for comparison, the choice of efficient micro-methods of assay and of a reliable base line to which one can refer one's results, may it be

wet weight, dry weight, protein-nitrogen, nucleoprotein-phosphorus, cell counts, all of which have advantages and disadvantages. There are the questions of varying contents of fat, of residual blood, of contamination with necrotic or inflamed tissue. Considering enzymes again—the orderly function of

TABLE 6. Gross Comparative Chemical Composition of Normal and Abnormal Tissue (after Winzler (1953))

Water	Significantly increased in most tumours as compared with homologous normal tissue.
Enzymes: respiratory	Warburg phenomenon, i e. increased glycolysis in tumours, enzyme systems, involved in aerobic oxidation, reduced.
Enzymes: catabolic, anabolic	Quantitative, but no qualitative differences, tumour enzyme pattern.
Metals and Trace Metals	Trend of increase of potassium in malignant tissues, clear tendency of decrease of calcium.
Other Proteins	Problem of protein changes as reflected by immuno-chemical tests.
	Increase in fibrous and collagenous material in some forms of abnormalities (fibromyomata, cheloids, etc.).
Nucleoproteins and Nucleic acids	[illegible]
Lipids, Phospholipids	Phospholipids seem to be increased in tumours.
Carbohydrates	Polysaccharides (mucoproteins) in serum of tumour-bearing hosts appear to be increased Changes of glycogen content of certain tissues.

which, according to Sumner and Myrback (1950) is one way of defining life, and their inhibition or hyper-function a manifestation of disease itself—attempts at establishing their true quantities in living material are beset with even more methodical devilries. It was not in a spirit of destructive criticism that Salter in 1944, after Greenstein had given a lecture (1944) on the admirable work done by him and his colleagues on enzyme distribution, asked him during the subsequent discussion whether one really measured their concentration if one assayed their activities in tissue slices or homogenates as Greenstein had done. It is well known that some of these

biological catalysts may exist under *in vivo* conditions in a bound or inhibited form, reduced or oxidized, aggregated or dissociated, complete or deficient in co-enzymes and activators. The assay of tissue extracts or slices may give somewhat misleading figures, because (*a*) the native functional proteins can change due to the influence of a host of variables during the estimation procedures, (*b*) the activity measurements do not disclose differences in distribution between various cellular particles, and (*c*) as indicated before, the results may not clearly demonstrate the existence of organ- and species-specific compounds catalysing the same reactions. However, when studying the findings of Greenstein (1954) for instance, (other laboratories have carried out similar work), with enzymes involved in amino-acid-, protein-, nucleic acid-metabolism and in respiratory processes, one cannot help noticing a certain trend which one should accept as a true one in spite of what was said just now. Thus, whilst no definite 'tumour enzyme' has so far been discovered, a tumour pattern has emerged. Following Warburg's work on the frequent predominance of anaerobic glycolysis in abnormal material, it has been stated by Greenstein that enzyme systems controlling aerobic oxidations are reduced in all tumours and that a number of others, at least in the rat and mouse, occupy usually a centre position between high and low activities as found in normal material. Of course, there is always the possibility that one of these days an enzyme will be found which is specific for normal or abnormal tissue.

Isocitritase has recently been discovered by Smith and Gunsalus (1954), true enough in a micro-organism, catalysing the production of succinic and glyoxylic acid, the latter, following Albert (1954), perhaps being capable of transforming purines into growth promoting pteridines; or there may exist specific alternative pathways of carbohydrate metabolism, like the direct oxidative one discovered by Dickens (1953) and established by him, Glock and others (1953) to be present in tumours; or it may be found, as Kaplan *et al.* (1954) have found with respect to an analogue of the coenzyme DPN which concentrates preponderantly in neoplastic tissues, that co-enzyme irregularities exist which have not yet been uncovered.

There hovers at the back of one's mind one big query. Are significant changes, if any, in the enzymic mosaic the cause or the consequence of abnormalities? This question can be raised in connection with the high contents of sulphatase and glucuronidase in urine from patients with bladder cancer as found by Boyland *et al.* (1954). It is well known that this malignant state develops, in a number of cases, following the carcinogenic action of dyestuff intermediates, such as β-naphthylamine, finally converted into the detoxicated sulphates and glucuronides of o-aminophenols. The presence or absence of enzymes catalysing the liberation of the carcinogenic phenols from these inert derivatives in the urine may be of importance, but whether the raised concentrations, particularly of glucuronidase, are associated with the development of the neoplasm or are due to its presence can only be decided by further investigations. In addition, other experiments are going on at the Chester Beatty Research Institute, which, apart from work done elsewhere, one rather optimistically believes, will, by improved techniques and more subtle approaches, help to disclose the true state of affairs. For instance, Dr. Lewin (1954) from New York, who is at present at the Institute, has started to follow the changes of xanthine oxidase, to come back once more to the first-mentioned enzyme, in normal breast tissue of female mice and to compare them with those occurring in spontaneous mammary tumours. Apart from direct enzyme assays, he will be looking into the question of tissue contents in molybdenum, which one will remember, forms an important part of the total enzyme molecule.

Metals and Trace Elements

This brings one to the general problem of the distribution in tissues of major and minor trace elements and particularly metals, a problem which at first sight appears to be easier to solve than that of enzyme contents. In addition to molybdenum a number of other trace metals have been found in course of time to be essential for the full activity of enzymes or coenzymes. Such enzymes include carboxypeptidase, in which only very recently zinc was discovered by Vallee and Neurath

(1954). Of course, there are other metal combinations in the body apart from the skeleton, the major ions, like Na, K, Ca and the iron in the haemoglobin of the erythrocytes; metal proteins such as ferritin and other complexes in salt—or chelate—form must play an important part in health and disease. How

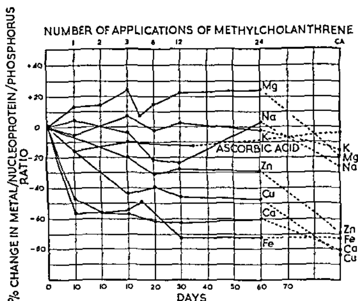


FIG. 2 Changes in the concentration of various components of the mouse skin in epidermal carcinogenesis induced by methylcholanthrene CA represents carcinoma. The dotted lines designate the change from late hyperplastic epidermis to carcinoma

do the concentrations of these metals behave in cases of abnormality? Figure 2 gives quantitative changes in the concentration of some metals in mouse skin treated with methylcholanthrene, according to Carruthers and Suntzeff (1945). The losses, as indicated, may be significant and, at least in the case of Ca, are, according to de Long *et al.* (1950), generally characteristic of cancer (see Table 6). These authors have also advanced the suggestion that this Ca-deficiency in tumour tissue is a factor involved in the decreased mutual adhesiveness of abnormal cells. While the skin-carcinoma of the mouse

shows slightly diminished potassium contents, other abnormal tissues, benign and malignant, disclose frequently an increase, as illustrated in the case of human breast material by a diagram in Hinsberg's book, *Das Geschwulstproblem* (1942). As different forms of bonding of these major and minor metals may contribute to their specific rôle and as this bonding could change their water- into fat-solubility, it is essential not to neglect the

TABLE 7. Analysis of Ignited Rat Tissue

K_2O	29,93	
Na_2O	8,19	
CaO	0,82	
MgO	2,15	
Fe_2O_3	0,04	Al_2O_3 < 02
SiO_2	0,11	
P_2O_5	48,21	
SO_3	0,69	
CO_2	0,44	
N_2O_5	0,40	
NaCl	2,97	
H_2O	1,02	
Organic matter	5,13	contains N and/or O
Total	100,10	

tissue lipids after their extraction from the total material when investigating tissues generally for metals. The difficulties which have been described when discussing enzyme assays, namely the selection of homologous tissue and of a reliable base line, etc. obtain also with this work; but here, the assay methods appear to be more easily controlled, even when extended to about 20–30 different elements. Everett and Martin are carrying out such experiments at the Chester Beatty Research Institute in collaboration with Webb of the Royal School of Mines (Bergel and Everett, 1953), and Miss Isobel Tipton and her colleagues (1954) in the United States have done so in a semi-quantitative way on normal human material. She discovered Cd in every kidney and Ti in every lung. The American and the London teams are using emission spectroscopy which has given such good results in soil and plant analyses. The ashed material is burned in a graphite spark and gives a line spectrogram. This

has the advantage of giving a large number of major and minor trace elements on one plate. A disadvantage is this: the line strength and thus the quantitative assessment of the minor trace elements depend to a certain extent on the ratio of the major inorganic constituents of the tissue ash. Unfortunately, not only do mouse and guinea pig differ in the composition of their main inorganic components (see Shohl, 1939) but, with regard to man, different age groups show considerable variations. So one has to analyse each time the reference tissue for these constituents, e.g. in the rat as given on Table 7 (by courtesy of Clements *et al.*, 1954), and to select the experimental material on the basis of same age, same sex, and same diet, including the drinking water—a tall order! Other methods of assay include the use of radioisotopes, a technique applied, for instance, by Wormall and colleagues (1953) with ^{65}Zn, neutron bombardments in an atomic pile, as done by atomic research stations here and in the States and of course, micro-colorimetric analyses. Whether the results will have any bearing on the causation of malignancy is a question that has to be left to the future, but it is hoped to obtain an idea as to the connection between cellular properties and the excess or deficiency of trace metals.

FINIS

Space is too short to say anything about differences between normal and abnormal tissues in proteins, other than enzymes, nucleoproteins and nucleic acids Their investigation is, of course, at least of the same importance as that of enzymes or trace elements On the contrary, an enormous amount of work has been done and is being done in various places, including the Chester Beatty Research Institute. Somerset Maugham said somewhere, 'I think one of the most useful discoveries I ever made was how easy it is to say "I don't know". I never noticed that it made anyone think the worse of me ' The few chemical data of normal and abnormal growth one knows, and even fewer which have been mentioned in this paper make it necessary to recall Maugham's statement.

REFERENCES

ALBERT, A. (1954). *Biochem. J.* **57**, x.

AVIS, P. G., BERGEL, F., BRAY, R. C. and SHOOTER, K. V. (1954). *Nature*, **173**, 1230

BERGEL, F., TIMMIS, G. M, FELTON, D. G, OSDENE, T. S., AVIS, P. G and BRAY, R. C. (1952). *B.E.C.C. Ann. Rep.* **30**, 58.

BERGEL, F. and EVERETT, J. L. (1953) *B.E.C.C. Ann. Rep.* **31**, 38.

BERGEL, F. (1953). *Nature*, **171**, 905.

BERGEL, F. (1954a). *Nature*, **174**, 156.

BERGEL, F. (1954b). *Med. Pr.* **232**, 214.

BOYLAND, E. (1954). *Lectures on the Scientific Basis of Medicine, Volume III: 1953–54.* The Athlone Press, London, p 327.

BOYLAND, E., WALLACE, D. M. and WILLIAMS, D. C. (1954). *Biochem. J.* **56**, xxix.

CARRUTHERS, C. and SUNTZEFF, V. (1945). *J. biol. Chem.* **159**, 647.

CLEMENTS, J. A., HARINGSHAW, J. F and THEOBALD, L S. (1954). Private communication.

[illegible]18.

[illegible] E C.C. *Ann. Rep* **31**, 79 ff.

DIETRICH, L. S. and SHAPIRO, D. M. (1953). *Cancer Res.* **13**, 699.

FEIGELSON, P., FEIGELSON, M. and WOOD, T. R (1954). *Science*, **120**, 502.

FRIEDRICH-FREKSA, H and ZAKI, F. G. (1954). *Z. Naturf.* **9b**, 394.

GREENSTEIN, J. P. (1944) Enzymes in Normal and Neoplastic Animal Tissue *A A A.S Res. Conf. on Cancer*, 192 ff., 216.

GREENSTEIN, J. P. (1954). *Biochemistry of Cancer*, 2nd ed, 431, 433. Academic Press, New York

HADDOW, A. (1935) *Nature*, **136**, 868.

HADDOW, A (1947) *Brit med Bull.* **4**, 331

HADDOW, A., HORNING, E S, TIMMIS, G. M., ROSS, W. C. J, SPICKETT, R. G. W. and AVIS, P. G (1951). *B.E C C. Ann Rep* **29**, 29.

HADDOW, A, HORNING, E. S, BERGEL, F, TIMMIS, G. M, OSDENE, T. S, BRAY, R. C., AVIS, P G and BROWN, A R (1953) *B E.C C. Ann. Rep.* **31**, 35

HADDOW, A. (1954) *Lectures on the Scientific Basis of Medicine*, 4th series, 1953–54. Not included in published volume.

HINSBERG, K. (1942) *Das Geschwulstproblem in Chemie und Physiologie* Steinkopff, Dresden.

HINSHELWOOD, C. (1952) *J chem Soc.* 745

KAPLAN, N. O, GOLDIN, A, HUMPHREYS, S R, CROTTI, M M and VENDITTI, J. M. (1954). *Science*, **120**, 437.

KLEIN, G. (1952). *Cancer Res* **12**, 589.

LEDOUX, L. (1955). *Nature*, **175**, 258.

LEWIN, I. (1954). Private communication
RICHERT, D. A., VANDERLINDE, R. and WESTERFELD, W. W. (1950). *J. biol. Chem* 186, 261.
SHOHL, A. T. (1939). *Mineral Metabolism*. American Chemical Society

Sc 54, 1177.
SUMNER, J. B. and MYRBACK, K. (1950). *The Enzymes*, vol. 1. Academic Press, New York.
TIPTON, I., STEINER, R. L., FOLAND, W. D , MUELLER, J. and STANLEY, M.

Yorl , p. 552.
WORMALL, A., TIPPER, R L. F. and WATTS, R. W. E. (1953). *B E.C.C. Ann. Rep.* 31, 114.

V

The Chemistry of the Porphyrias

C. H. GRAY

It is the purpose of this lecture to illustrate how the chemical investigation of a relatively rare group of diseases, the porphyrias, has developed side by side with fundamental investigations of the biosynthesis of haem of the haem proteins and how progress in one field has been greatly accelerated by developments in the other. I therefore propose to give a brief account of the clinical aspects of the porphyrias, to outline our knowledge concerning the biosynthesis of porphyrins, to give an account of some very recent work carried out in this field and finally to discuss some possible mechanisms of the derangement of metabolism in these diseases.

The porphyrias are essentially diseases accompanied by a primary abnormality of porphyrin metabolism and are distinguished from the porphyrinurias in which there is an increased production of normally occurring porphyrins secondary to some other disease. They have been classified by Waldenström (1937) into three types, acute, congenital and a third form—porphyria cutanea tarda.

In the acute form of the disease, or acute intermittent porphyria as it is called by Watson (1954), the disease characteristically first becomes manifest in early adulthood and is often precipitated as an acute crisis following the administration of sulphonamides or barbiturate for some other condition. Attacks of abdominal colic with constipation are accompanied by lesions of the central nervous system which may be very varied in character. These lesions take the form of a severe peripheral neuritis with paralyses, and there is often central and even

cerebral involvement as well, with the development of frank psychoses. This clinical picture is very variable, some cases having abdominal symptoms only, while others are referable mainly to the central nervous system. Many cases have been operated upon because a surgeon has suspected an intestinal obstruction. Others have been regarded as examples of some obscure neurological disease, and many have been found in mental hospitals or attending psychiatric clinics. It is probable that with more widespread knowledge of the clinical features of the disease such wrong diagnoses will become less frequent.

In congenital porphyria (light-sensitive porphyria—Watson) there are no abdominal or central nervous system lesions. Instead there is severe photosensitivity beginning at birth or in infancy and of such a severity that the skin and the underlying tissues of the exposed parts become scarred and grossly deformed (Plate II, Figure 1*a*).[1] Porphyrins are deposited in the tissues and result in intense pigmentation of the bones, teeth and skin.

Porphyria cutanea tarda (mixed porphyria according to Watson) is said to be rarer but is probably less frequently recognized. It takes the form of a mild acute intermittent porphyria with abdominal symptoms but not usually central nervous system lesions. In addition there is a mild photosensitivity which, however, does not lead to the severe scarring and deformities so characteristic of the congenital form of the disease (Plate III, Figure 1*b*). It is often associated with sensitivity to trauma, the development of the skin lesions often conditioned by an abrasion of an exposed part (Plate IV, Fig. 1*c*). In some of these cases there is jaundice present and then the photosensitivity may be more severe (Plate V, Figure 1*d*).

Porphyrinuria is essentially secondary to other disease, such as liver dysfunction, pernicious anaemia, lead poisoning, haemolytic anaemia or poliomyelitis (Watson and Lawson, 1947). The increased excretion of porphyrins in these conditions is not usually great and as far as is known there are no symptoms referable to that excretion.

Before passing to the chemical findings in porphyria, I should like to draw attention to the general interrelationship of the

[1] The plates referred to in this lecture will be found between pages 80–1.

porphyrins themselves. It is almost certain that all naturally occurring porphyrins are derived from uroporphyrins. These are tetrapyrrolic pigments in which the four pyrrole rings are

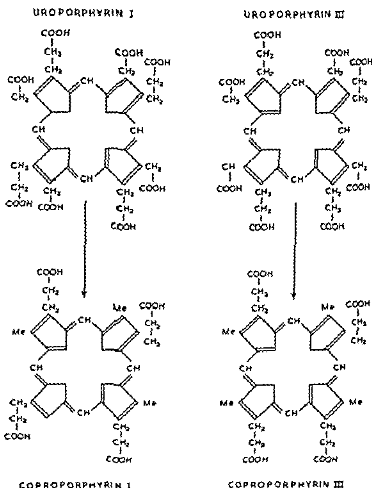


FIG. 2 Structure of uroporphyrins and coproporphyrins I and III.

united by methene (CH) groups to form one large ring-structure. Four acetic-acid groups and four propionic-acid groups occupy the β positions in the pyrrole rings Only four arrangements of these groups are possible and only two of these four

arrangements have so far been found in nature. These are the arrangements found in uroporphyrin I excreted in congenital porphyria and the arrangement in uroporphyrin III which occurs as a copper complex in turacin, the wing pigment of the tropical bird turacao. In uroporphyrin I the acetic- and propionic-acid groups are arranged alternately round the ring but in uroporphyrin III the groups attached to one pyrrole ring have been interchanged. Uroporphyrins II and IV with other arrangements have so far never been demonstrated in nature.

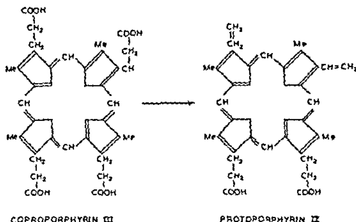


Fig. 3. Relationship of coproporphyrin III to protoporphyrin.

Uroporphyrins I and III may be decarboxylated with the loss of four molecules of carbon dioxide to give the corresponding coproporphyrins I and III, the decarboxylation converting the acetic-acid groups into methyl groups (Figure 2). In the body, coproporphyrin III is probably oxidatively decarboxylated with loss of two molecules of carbon dioxide and four hydrogen atoms from two of the propionic-acid groups to form protoporphyrin IX in which the side chains consist of four methyl, two vinyl and two propionic-acid groups (Figure 3). The iron complex of this protoporphyrin IX is the prosthetic group of most haem proteins such as haemoglobin, myoglobin, cytochrome c, peroxidases and catalase.

porphyrins themselves. It is almost certain that all naturally *occurring porphyrins* are derived from uroporphyrins. These are tetrapyrrolic pigments in which the four pyrrole rings are

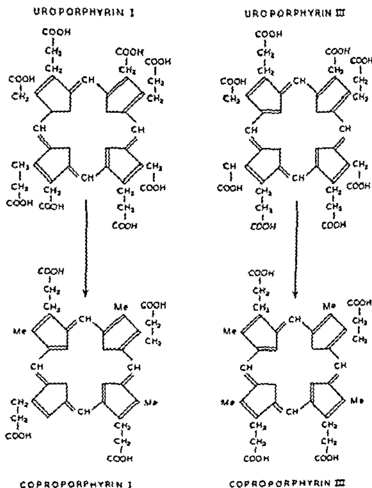


FIG 2 Structure of uroporphyrins and coproporphyrins I and III.

united by methene (CH) groups to form one large ring-structure. Four acetic-acid groups and four propionic-acid groups occupy the β positions in the pyrrole rings Only four arrangements of these groups are possible and only two of these four

porphyrin containing 8 carboxyl groups in its molecule and which perhaps may be uroporphyrin III.

Isotope Studies in Congenital Porphyria

In 1948, three independent groups of workers in London, New York and Minneapolis made use of glycine labelled with ^{15}N, the heavy isotope of nitrogen, for the study of congenital porphyria (Gray and Neuberger, 1950; London, West, Shemin and Rittenberg, 1950a, 1950b; Grinstein, Aldrich, Hawkinson, Lowry and Watson, 1951).

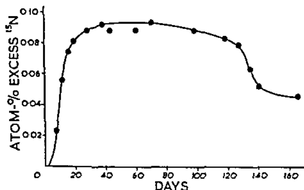


Fig. 4. Concentration of ^{15}N in haemin samples of a normal subject who had been given [^{15}N]-glycine Labelled glycine [12 g], containing 31 65 atom-per cent excess ^{15}N, was given during days 1–4

Shemin and Rittenberg (1945, 1946a) had already shown that glycine was a specific precursor of the haem of haemoglobin and had found that when ^{15}N labelled glycine was fed to a normal human subject (Shemin and Rittenberg, 1946b) the ^{15}N content of the haem of the haemoglobin changed with time, as is shown in Figure 4. The shape of the curve showed that haemoglobin was not continuously synthesized and degraded like the other body proteins, but that the haemoglobin had a definite life-span corresponding to that of the red cells (Figure 4).

Soon after the glycine feeding, the increase in isotope in the red cells with time represents the passage of newly formed

The Chemistry of the Porphyrias

Until about 1945, the chemistry of the porphyrias seemed quite straightforward. In acute intermittent porphyria, while the faeces contained coproporphyrin III, the urine contained this same coproporphyrin III together with much larger amounts of uroporphyrin III, partly as a metal complex and partly as a colourless precursor porphobilinogen. This precursor, readily detectable by the red colour given with Ehrlich's aldehyde reagent, was converted into uroporphyrin III and a brown pigment porphobilin when the urine was allowed to stand in light (Waldenstrom, 1937). In contrast, in congenital porphyria, the faeces contain large amounts of coproporphyrin I which was also present in the urine together with much larger amounts of uroporphyrin I.

Table 1 Views concerning the nature of urinary porphyrins in acute porphyria

1	Uroporphyrin III (Waldenström, 1937)
2	Uroporphyrin III with uroporphyrin I (Mertens, 1937)
3	Uroporphyrin I and unidentified porphyrins (Grinstein, Schwartz and Watson, 1945)
4	Mainly uroporphyrin III with uroporphyrin I and porphyrins of intermediate carboxylation as minor constituents (Nicholas and Rimington, 1953)

This simple view that excretion of type III porphyrins or their derivatives was characteristic of acute porphyria, while type I porphyrins were excreted in the congenital form of the disease is now known to be incorrect (Table 1). Uroporphyrin I sometimes accompanies uroporphyrin III in acute porphyria (Mertens, 1937). So-called uroporphyrin III may consist of a mixture of porphyrins including uroporphyrin I (Nicholas and Rimington, 1953), which is sometimes present in [illegible] amounts (Grinstein, Schw[illegible] Harri[illegible] po[illegible] obtained from the po[illegible] heating the acidified urine. The uroporphyrin I of congenital porphyria has been found to contain small amounts of porphyrins containing 7, 5 and 4 carboxyl groups (Rimington and Miles, 1951) as well as another

PLATE II



FIG. 1a Congenital porphyria (Reproduced, by permission of John Wright & Sons Bristol, from French, *Index to Differential Diagnosis*.)

labelled red cells into the circulation. These labelled red cells replace cells in process of destruction, which were formed before administration of the glycine and are hence unlabelled. After the glycine feeding, the ^{15}N content of the body glycine decreases in an exponential manner and when it reaches insignificant levels the newly formed red cells will again become unlabelled. They will still be replacing the unlabelled red cells formed before the glycine feeding and the curve becomes practically horizontal. Finally the labelled cells reach the end

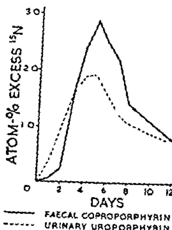


FIG. 5. Concentration of ^{15}N in faecal coproporphyrin and urinary uroporphyrin of a patient with congenital porphyria who had been given [^{15}N]-glycine Labelled glycine [12 g], containing 31 65 atom-per cent excess ^{15}N, was given during days 1–4.

of their life-span and will begin to be destroyed. Since they will now be replaced by newly formed unlabelled red cells, the ^{15}N in haemin declines When following the incorporation of ^{15}N into the haem of haemoglobin, newly formed labelled haem is extensively diluted with preformed unlabelled haem and the ^{15}N content of the newly formed haem can only be calculated indirectly by mathematical analysis. This dilution with previously formed unlabelled material would not be expected with the porphyrins excreted by a case of congenital porphyria if it is assumed that the porphyrins of the series I



FIG 1c Traumatic blister in *porphyria cutanea tarda* (Reproduced, by permission of the editors, from the *Quarterly Journal of Medicine*, vol **17**)

PLATE III



FIG. 16. *Porphyria cutanea tarda* (Reproduced by permission of the editors, from the *Quarterly Journal of Medicine* vol 17)

and III have part of their metabolic pathway in common, in other words that glycine should be the precursor of the nitrogen atoms of coproporphyrin I and uroporphyrin I. ^{15}N glycine was therefore fed to a case of congenital porphyria and, at suitable time intervals, there were determined the ^{15}N contents of the urinary and faecal porphyrins, of the haem of circulating haemoglobin and of the faecal stercobilin. Figure 5 shows the incorporation of ^{15}N into urinary uroporphyrin and faecal coproporphyrin. The faecal coproporphyrin labelling rose

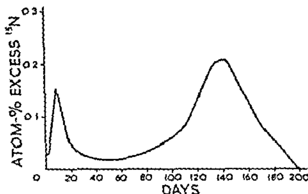


FIG. 6. Concentration of ^{15}N in stercobolin hydrochloride of a normal subject who had been given [^{15}N]-glycine. Labelled glycine [12 g], containing 31 65 atom-per cent excess ^{15}N, was given during days 1–4

quickly to a maximum the day after glycine feeding had ended. The urinary uroporphyrin labelling rose even more quickly to a somewhat lower maximum, perhaps because the kidney provided a faster route of excretion than the gut. It appeared therefore that the labelled glycine is incorporated into the porphyrin very quickly indeed. The lower labelling of the uroporphyrin suggested that there was some dilution of the porphyrin with preformed unlabelled material. This would be in keeping with the known storage of uroporphyrin in the bones, liver and teeth. Mathematical analysis of all the data showed that in congenital porphyria, coproporphyrin I, uroporphyrin I and protoporphyrin of the circulating haem are derived from a common precursor.

PLATE V



Fig. 1d Photosensitivity in patient with *porphyria cutanea tarda* and jaundice

only about 15 per cent of the excreted bile pigment instead of about 80 per cent. Most of the bile pigment in congenital porphyria is formed from glycine by a very rapid mechanism. Infra-red analysis, elemental analysis, etc., showed that the stercobilin from congenital porphyria is the same as normal stercobilin so that this pigment in congenital porphyria is not derived from type I porphyrin nor can it have uro-type side chains.

Biosynthesis of Porphyrins

It is useful at this stage to consider what is now known regarding the mechanism of synthesis of porphyrins. Using glycine labelled with ^{15}N and with ^{13}C or ^{14}C in the methylene or carboxyl group and acetic acid labelled in either the methyl group or the carboxyl group, it has been possible to show the origin of every atom in the protoporphyrin molecule. In these studies, Muir and Neuberger (1950) have used the intact animal while Shemin and Wittenburg (1951) have used duck erythrocytes which actively synthesize haem *in vitro*. The protoporphyrin molecule is synthesized from acetic acid and glycine only (see Figure 8*a*).

If, as appeared probable, protoporphyrin is formed by decarboxylation and dehydrogenation of uroporphyrin III the source of the carbon atoms of uroporphyrin III are by inference as shown in Figure 8*b*. All four of the pyrrole rings appeared to be similarly synthesized and porphyrin synthesis would most simply be accounted for by the condensation of four monopyrrole units bearing a potential methene group in an α position and an acetic-acid and a propionic-acid side chain in the β position.

Shemin and Kumin (1952) produced important evidence that two molecules of an asymmetrical succinyl derivative condense with glycine to form the monopyrrole unit of the desired type (Figure 9). Such an asymmetrical succinyl derivative would be formed via the Krebs cycle from two molecules of acetic acid and would account for two of the carbon atoms in each of the pyrrole rings being formed partly from the methyl group and partly from the carboxyl group of acetic acid. Thus the biosynthesis of uroporphyrin would take place by the

Before considering the incorporation of ^{15}N into the stercobilin of the patient with congenital porphyria it is necessary to show the stercobilin curve for a normal individual (Figure 6).

The stercobilin ^{15}N curve shows two peaks: (1) immediately after glycine feeding and (2) with a maximum about the 130th day, when the declining haem curve shows labelled red cells are breaking down. There is also small but significant labelling between these two peaks. These results have been interpreted

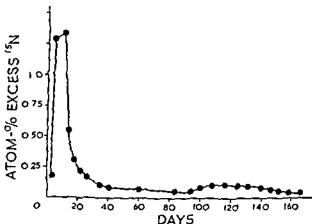


FIG 7 Concentration of ^{15}N in stercobilin hydrochloride of a patient with congenital porphyria who had been given [^{15}N]-glycine. Labelled glycine [12 g], containing 31 65 atom-per cent excess ^{15}N, was given during days 1–4.

by assuming three metabolic sources of stercobilin: (1) from cells at the end of a normal life-span; (2) by a very rapid mechanism at first thought to be by direct synthesis, but now believed to be from protoporphyrin synthesis in excess of globin formation in the bone marrow; (3) by a slow mechanism *not* from the cells at the end of a normal life-span.

A very different picture was obtained in congenital porphyria (Gray, Neuberger and Sneath, 1950; London, West, Shemin and Rittenberg, 1950a, 1950b). Figure 7 shows how the first peak was greatly exaggerated while the second peak was barely noticeable. In congenital porphyria therefore, breakdown of red cells at the end of their normal life-span is responsible for

(Figure 8*c*) to give uroporphyrin (Figure 8*b*). This then would be decarboxylated to form protoporphyrin (Figure 8*a*).

It has now been established beyond doubt that the monopyrrolic intermediate is in fact porphobilinogen, the colourless precursor excreted in acute porphyria. Porphobilinogen was

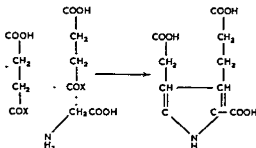


FIG. 9. Condensation of two molecules of an asymmetric derivative of succinic acid with glycine (Shemin and Kumin).

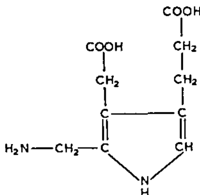
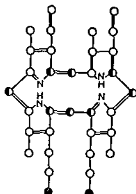
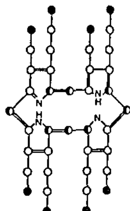


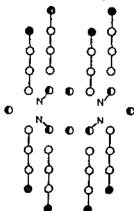
FIG. 10. Porphobilinogen.

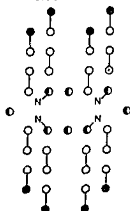
first isolated by Westall (1952), and Cookson and Rimington (1954) have shown that its structure is that of a monopyrrole with acetic- and propionic-acid side chains, but with an aminomethyl group in the α position (Figure 10). It is readily converted in acid solution into uroporphyrin III together with traces of other porphyrins. In the avian red-cell system *in vitro* it is

condensation of two carbon atom units (Figure 8*d*) to form four carbon atom derivatives which would unite with glycine









○ METHYL GROUP OF ACETIC ACID
● CARBOXYL GROUP OF ACETIC ACID
◐ ∝-CARBON OF GLYCINE
○ METHYL & CARBOXYL GROUPS OF ACETIC ACID

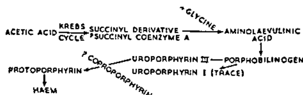
FIG. 8. Porphyrin biosynthesis (after Shemin).

porphobilinogen, uroporphyrin, coproporphyrin and protoporphyrin by avian red-cell haemolysates (Dresel, 1955).

Shemin and Russell (1953) and Neuberger and Scott (1953) have shown that δ-aminolaevulinic acid is a precursor of haem. They were looking for the asymmetrical succinyl derivative suggested by Shemin three years before and had prepared the amino-β-keto adipic acid which may be regarded as glycine succinylated at the methylene carbon atom (Figure 11). This is readily decarboxylated to give δ-aminolaevulinic acid, two molecules of which can condense to form porphobilinogen (Figure 12).

^{14}C labelled δ-aminolaevulinic acid is converted to haem in the rat and to porphobilinogen, uroporphyrin, coproporphyrin and protoporphyrin in the fowl red-cell system.

We thus see that the general pathway of synthesis of haem is as follows:



Metabolic Abnormalities in Porphyrias

It is possible to postulate probable sites of interference with porphyrin synthesis in the various forms of porphyria (Muir, 1954).

In congenital porphyria there is a genetically determined abnormality of enzymic conversion of porphobilinogen to types I and III porphyrins in the bone marrow (Figure 13). Normally the daily output of type I porphyrins is only a few hundred μgs. and therefore readily excreted. In congenital porphyria. the amounts of type I porphyrins are very great and may amount to 100 mg. per day. These type I porphyrins are useless for synthesis of prosthetic groups and they are not degraded to bile pigments. They are therefore excreted or deposited in the body, a well-recognized feature of the disease. The photosensitivity is almost certainly due to the photodynamic effect

converted enzymically to protoporphyrin and uroporphyrin. Under anaerobic conditions, coproporphyrin is found together with an increased amount of uroporphyrin suggesting that coproporphyrin might be a normal intermediate in the conversion of uroporphyrin to protoporphyrin (Falk, Dresel and Rimington, 1953).

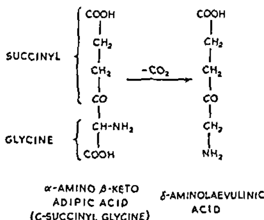


FIG. 11. Decarboxylation of α-amino β-keto adipic acid to δ-amino-laevulinic acid.

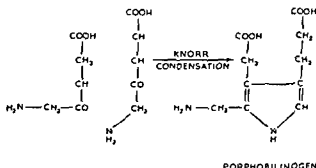


FIG. 12. Conversion of δ-aminolaevulinic acid to porphobilinogen.

That porphobilinogen itself is a normal intermediate has been shown by the conversion of ^{14}C glycine into labelled

because of the block in synthesis. In human acute porphyria there is no fall in the liver catalase and it seems probable that porphobilinogen formation is in excess of that required for normal haem synthesis. Gray, Neuberger and Scott (1955) have attempted to look into this using δ-aminolaevulinic acid. Berlin, Neuberger and Scott (1955) have shown that δ-aminolaevulinic acid on administration to normal man is converted into urinary porphobilinogen, faecal protoporphyrin and causes an erythema of the exposed parts. When ^{14}C labelled material is administered, the carbon dioxide, the faecal protoporphyrin and stercobilin, and the urinary porphobilinogen are all highly labelled. Although not all the labelled carbon was accounted for, the degree of labelling was consistent with the conversion of porphobilinogen to protoporphyrin and stercobilin in that order and the measurements of the dilutions of these compounds in the body suggested that there are small pools of the δ-aminolaevulinic acid and the porphobilinogen The size of these pools could not be estimated accurately because of slow equilibration and rapid excretion, but a rough estimate showed that in all forms of porphyria the pools, or more strictly the dilution capacities, of δ-aminolaevulinic acid and of porphobilinogen were considerably increased. Moreover, in acute porphyria the conversion of δ-aminolaevulinic acid to porphobilinogen is greatly increased and it seems thus possible porphyria is associated with either an increased amount of δ-aminolaevulinic acid formed from glycine and succinic acid, or a decreased metabolism via pathways other than via porphobilinogen. Perhaps the high conversion of δ-aminolaevulinic acid to porphobilinogen is related to an increase in the enzymic activity responsible for this conversion. It is clear that further experiments are required before we know the precise lesion in acute porphyria.

Neither porphobilinogen nor δ-aminolaevulinic acid has any obvious pharmacological effect on the central nervous system. It seems likely that lesions of the nervous system in acute porphyria are due not so much to a toxic action of some compound but to some enzymic abnormality in the glycine-succinate metabolism Such a view would account more readily

of the free porphyrins in the tissues which are known to be associated with the release of histamine. Aldrich, Hawkinson, Grinstein and Watson (1951) have shown the presence of free uroporphyrin I in the red cells and it is not impossible that haemolysis of the red cells, which is such a characteristic feature of the condition as has been apparent on clinical grounds (Guenther, 1925) as well as demonstrated by the isotope technique (Gray, Muir and Neuberger, 1950), is due to photosensitized haemolysis. This increased rate of haemolysis is normally compensated by corresponding increase in synthesis

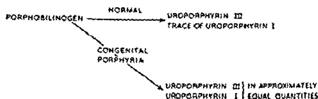


FIG 13 Abnormality in congenital porphyria.

of haem; anaemia in this condition is uncommon. This increased synthesis of haem aggravates the condition producing increased quantities of uroporphyrin I which is a by-product of that synthesis. Splenectomy is sometimes effective in reducing the rate of haemolysis in certain cases, and by reducing the rate of haemoglobin synthesis should result in a diminution of excretion of porphyrins. The operation was very successful in the case described by Aldrich *et al.*, but no permanent benefit, such as there was being in any case very slight, was observed in the case described by Gray and Neuberger (1952).

In acute porphyria there is no photosensitivity, presumably because the free porphyrins are present only in traces in the tissues. The essential feature is the excretion of porphobilinogen but the precise nature of the porphyrins excreted seems to be unimportant. It is possible that there is a block in the conversion of porphobilinogen to porphyrins and indeed in the experimental porphyria induced by sedormid, Schmidt and Schwartz (1952) showed that there was a diminution in the catalase formation in the liver and they postulated that in this form of porphyria excess porphobilinogen is excreted in the urine

SHEMIN, D. and KUMIN, S. (1952). *J. biol. Chem* **198**, 827.
SHEMIN, D. and RITTENBERG, D (1945). *J. biol. Chem.* **159**, 567.
SHEMIN, D and RITTENBERG, D (1946a). *J. biol. Chem.* **166**, 621.
SHEMIN, D. and RITTENBERG, D (1946b). *J. biol. Chem.* **166**, 626.
SHEMIN, D. and RUSSELL, C. S. (1953). *J. Amer. chem. Soc.* **76**, 4873.
SHEMIN, D. and WITTENBURG, J. (1951). Ciba Foundation Conference on Isotopes in Biochemistry, p 41. J. and A Churchill, Ltd., London.
WALDENSTRÖM, J. (1937). *Acta med. Scand. Suppl.* 82.
WATSON, C. J. (1954). *Advances in Internal Medicine*, vol. vi. Year Book Publishers Inc., Chicago.
[illegible] Urinary Coproporphyrin in

for the extremely patchy and diverse nature of the clinical lesions in the disease.

It is not wise to speculate on the nature of porphyria cutanea tarda in the present state of knowledge, although it is tempting indeed to speculate on the significance of the erythema, resulting from the administration of δ-aminolaevulinic acid to normal man.

REFERENCES

ALDRICH, R A., HAWKINSON, V., GRINSTEIN, M. and WATSON, C. J. (1951). *Blood*, 6, 685.

BERLIN, N. I., NEUBERGER, A. and SCOTT, J. J. (1955). *Biochem. J.* in press.

[illegible]

ed. G. E. W. Wolstenholme. J. and A. Churchill, Ltd., London.

FALK, J. E , DRESEL, E. I. B. and RIMINGTON, C. (1953). *Nature, Lond.* 172, 292.

GIBSON, Q. H. and HARRISON, D. C. (1950). *Biochem J.* 46, 154.

GRAY, C. H., MUIR, H. M. and NEUBERGER, A. (1950). *Biochem. J.* 47, 542.

GRAY, C. H. and NEUBERGER, A (1950). *Biochem. J.* 47, 81.

GRAY, C H. and NEUBERGER, A (1952) *Lancet*, 1, 851.

GRAY, C. H., NEUBERGER, A. and SCOTT, J J. (1955). *Biochem. J.* in press.

GRAY, C H., NEUBERGER, A and SNEATH, P. H. A. (1950). *Biochem. J.* 47, 87.

GRINSTEIN, M., ALDRICH, R. A., HAWKINSON, V., LOWRY, P. and WATSON, C. J. (1951). *Blood*, 8, 699.

GRINSTEIN, M., SCHWARTZ, S. and WATSON, C J (1945). *J biol Chem.* 157, 323.

GUENTHER, H. (1925). *Handbuch der Krankheiten des Blutes und der Blutbildenden Organe*, vol 2. Berlin.

LONDON, I. M., WEST, R , SHEMIN, D. and RITTENBERG, D. (1950a). *J. biol. Chem* 184, 351.

LONDON, I M , WEST, R., SHEMIN, D. and RITTENBERG, D. (1950b). *J. biol. Chem* 184, 365

[illegible] *Chemical Pathology*

[illegible] 97

[illegible] 55, 109.

[illegible] 2

[illegible] 40, 939.

Geneticists have established from the study of the blood groups in families that the group characters A and B and their absence—called group O—are inherited according to Mendelian laws and it is now generally believed that the blood group of the individual depends on the presence of two of three allelomorphic genes (called A, B or O) any one of which is capable of occupying the same locus on a chromosome.

TABLE 1

Blood group of person	Specific agglutinable substance in the erythrocytes	Specific iso-agglutinin in the serum
A	A	anti-B (β)
B	B	anti-A (α)
AB	A and B	—
O	O	anti-A (α) and anti-B (β)

TABLE 2

Gene from one parent	Gene from other parent	Combination of genes [genotype]	Serologically demonstrable blood group [phenotype]
A	A	AA	A
A	O	AO	A
B	B	BB	B
B	O	BO	B
A	B	AB	AB
O	O	OO	O

In any person half the germ cells (the gametes) carry one blood group gene and the other half carry the other blood group gene, thus an AB person produces A and B gametes in equal numbers, AO produces A and O in equal numbers, etc., while homozygous persons AA, BB and OO yield only germ cells of one kind A or B or O. It is evident, therefore, that no [illegible]

mating in Figure 1.

VI

The Chemical Basis of Blood Group Specificity in Man

W. T. J. MORGAN

LANDSTEINER, at the beginning of the century, was the first to show that serum and erythrocyte specimens obtained from different persons frequently gave, when mixed together, a clumping or agglutination of the erythrocytes. *In some instances, however, no agglutination took place.* It was found that, according to whether or not the red cells contain one or two factors (designated quite arbitrarily as A or B) or neither factor, human bloods could be classified into four groups. The serologically specific agent in the serum—the agglutinin—which brings about the clumping of the red cells occurs naturally and Landsteiner concluded that *a person's serum cannot contain antibody for antigens present in his own* erythrocytes. Thus a definite relationship between different kinds of human blood was discovered and subsequently developed into what is now known as the ABO blood group system (Table 1). There are, of course, many other blood group systems but here we are concerned with the ABO system, with only a passing reference to one other blood group specificity.

It is to avoid an agglutinable substance on the surface of the [illegible] during [illegible] which exists [illegible] hat an understanding of the serological reactivities within the ABO *system is indispensable. This is one of the most important* clinical aspects of blood transfusion, a technique which plays such an important role in modern medical practice.

Geneticists have established from the study of the blood groups in families that the group characters A and B and their absence—called group O—are inherited according to Mendelian laws and it is now generally believed that the blood group of the individual depends on the presence of two of three allelomorphic genes (called A, B or O) any one of which is capable of occupying the same locus on a chromosome.

TABLE 1

Blood group of person	Specific agglutinable substance in the erythrocytes	Specific iso-agglutinin in the serum
A	A	anti-B (β)
B	B	anti-A (α)
AB	A and B	—
O	O	anti-A (α) and anti-B (β)

TABLE 2

Gene from one parent	Gene from other parent	Combination of genes [genotype]	Serologically demonstrable blood group [phenotype]
A	A	AA	A
A	O	AO	A
B	B	BB	B
B	O	BO	B
A	B	AB	AB
O	O	OO	O

In any person half the germ cells (the gametes) carry one blood group gene and the other half carry the other blood group gene; thus an AB person produces A and B gametes in equal numbers; AO produces A and O in equal numbers, etc., while homozygous persons AA, BB and OO yield only germ cells of one kind A or B or O. It is evident, therefore, that no [illegible]

mating in Figure 1.

When genes *A* or *B* are transmitted with gene *O* at fertilization, character A or B alone is serologically demonstrable. Genes *A* and *B* are said to be 'dominant' to gene *O* or, put another way, *O* is recessive to the genes *A* or *B*. The homozygote, *AA*, and the heterozygote, *AO*, both belong to group A and no serological means are known to distinguish *for certain* the homozygous from the heterozygous condition.

The genetical and immunological basis of the specificity of human blood group characters is, especially within the ABO classification, firmly established and it is now generally accepted

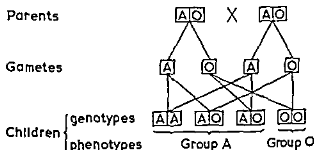


Fig. 1 Segregation of the blood group characters among the children of two heterozygous (AO) persons

that the group properties of the erythrocytes arise from the presence on their surface of substances which are products of the activity of the blood group genes.

The A, B and so-called O substances are secreted in a water-soluble form by about 80 per cent of all persons. The soluble material secreted by group O persons, however, is not an exclusive product of the activity of the *O* gene, as the A and B substances are the special products of the activities of the *A* and *B* genes. Persons belonging to group O secrete a substance which neutralizes a large number of reagents of human, animal and plant origin which agglutinate group O cells preferentially, hence the reason for the active substance being termed O-substance. The same substance is, however, secreted by persons belonging to groups A and B as well as by those of genotype A,B and these latter individuals do not possess an *O* gene according to Bernstein's theory of blood group inheritance

which is now generally accepted. To avoid confusion it has been suggested (Morgan and Watkins, 1948) that the mucoid material secreted by a group O person should be designated H substance as this would differentiate it as a heterogenetic, basic or primary substance common to the great majority of red cells, tissue fluids and secretions irrespective of the ABO group of the individual. The mucoid in the secretions from group O persons is therefore now referred to as H substance and not as O substance.

There remain about 20 per cent of individuals who do not secrete A, B or H specific substances in a water-soluble form. It has been shown that these persons secrete a blood group factor called 'Lewis' which is very similar chemically to the A, B and H substances and is a product of a gene Le^a which belongs to another blood group system. In just a few instances, probably less than 1 per cent of all persons, individuals fail to secrete any of the known blood group substances including the second Lewis agglutinogen, Le^b. It must be emphasized, however, that our knowledge of the 'Lewis' system of blood groups is far from complete and that perhaps these individuals are secreting an unidentified 'Lewis' factor. The point to be made here is that they certainly secrete a mucoid material of some kind, as can be readily demonstrated.

Human sera which agglutinate group O cells preferentially are occasionally found in man and some, but not all, of these sera are neutralized by H substance. The red cells of some individuals fail to react with strong H agglutinins and the erythrocytes of these persons are considered to be devoid of H substance (Bhende, Deshpande, Bhatia, Sanger, Race, Morgan and Watkins, 1952). This finding seems to indicate that H substance is not a mere species antigen common to all human beings. However, it cannot be accepted unreservedly as a blood group agglutinogen, as are the A and B substances, until the inheritance of the H character has been established by family studies.

With this broad and rather superficial account of the blood group relationships we can now consider the isolation of the materials which are responsible for the characteristic serological behaviour of the erythrocytes, tissue fluids and secretions.

THE ISOLATION OF GROUP SPECIFIC SUBSTANCES

The earliest attempts to isolate the group substances were made by extracting erythrocytes with ethanol and other organic solvents. The products obtained were of low activity and were never satisfactorily purified, but the results established that the specific substances were most probably of carbohydrate nature. There are special difficulties associated with the isolation of group substances from erythrocytes for it appears that the specific material is bound in some way with the lipid and possibly protein constituents of the red-cell surface and is in consequence practically insoluble in water, and even today little is known concerning the exact chemical nature of the blood group specific complexes on the erythrocyte surface (cf. Morgan, 1947).

A differential inactivation of certain group specific receptor sites on the erythrocyte can be brought about by oxidation with the periodate ion (Morgan and Watkins, 1951) and in some instances group specific receptors on the red-cell surface can be destroyed by enzymes (Watkins and Morgan, 1954). The results of experiments of this kind offer additional and indirect evidence that within the ABO and Lewis blood group systems the substances on the erythrocyte surface responsible for the specific serological characters are most probably of carbohydrate nature.

A large amount of work on the isolation and characterization of water-soluble group substances has been carried out since the time of their discovery about twenty-five years ago, and most workers reported that the active substances were carbohydrates but that they were unable to distinguish chemically between the preparations which possessed A, B or the so-called O activity. Indeed it would be fair to say that no description of a group substance which could be considered substantially free from contaminating unspecific material was reported until a few years ago. The small yield of group substances obtained from gastric juice and saliva, the most potent sources of water-soluble blood group materials known until recently, prompted an examination of ovarian cyst fluids for their content of specific substances and revealed that these pathological overgrowths are a potent source of group specific material and are

frequently found to contain considerable amounts of active substance (Morgan and van Heyningen, 1944). More recently *meconium, the first stool of the newborn, has also been found* to be rich in group specific material (Buchanan and Rapoport, 1951).

Many methods for the isolation and purification of group substances have been described but it has generally been found that extraction of the freeze-dried tissue fluid or secretion with cold 90 per cent phenol is a very efficient method for eliminating the major part of the protein impurities. This treatment leaves behind as a phenol-insoluble residue a predominantly mucoid material which possesses almost the whole of the specific activity of the original secretion. The active substances can then be further purified by high-speed centrifugation or fractionated from water and certain organic solvents and obtained in an apparently homogeneous condition. It is advisable to avoid during isolation of the group substance high temperatures and all reagents which are strongly acid or alkaline in reaction The use of these restricted conditions for the manipulation of the large labile molecular species which carry the blood group specific characters, allows certain irreversible changes to be avoided or reduced to a minimum and in consequence it is possible to obtain these group materials in quantities suitable for chemical study and at the same time largely in their unchanged or 'native' state. Full details of the procedures employed to isolate the group substances from human ovarian cyst fluids have been given (Morgan and Waddell, 1945; Aminoff, Morgan and Watkins, 1950; Annison and Morgan, 1952a, b; and Gibbons, Morgan and Gibbons, 1955) and will not be described here.

The next problem is to satisfy oneself that the material obtained is substantially homogeneous on the basis of physical, chemical and immunological measurements. If any significance is to be attached to the subsequent isolation of a particular sugar or amino-acid, which is present in small amounts only, the homogeneity of the preparation of the blood group substances studied is of prime importance. This is a point which is frequently overlooked. Unfortunately, it is exceedingly difficult to

establish the homogeneity of mucoid materials of this kind and that is why so frequently in the past no serious attempt was made to do so and relatively crude materials only were used in chemical and immunological studies.

In our own studies we have usually required that the group materials should show no evidence of inhomogeneity on electrophoresis and ultracentrifugation at pH 4 and 8, that they should give constant analytical figures when examined by fractional solubility tests and should show an absence of appreciable amounts of other known serological blood group activities. Kabat and his co-workers (Kabat and Bezer, 1945; Kabat, 1949, 1952) have provided an excellent method, based on the specific precipitation of the group materials with the corresponding immune serum, for obtaining evidence as to the homogeneity of preparations of the group substances.

The Properties of the Group Substances

Physical. The examination by physical methods (Kekwick, 1950, 1952a, b; Caspary, 1954) of carefully purified preparations of the group substances shows that the materials possess a remarkable degree of homogeneity. Their behaviour during electrophoresis at pH's 4·0 and 8·0 indicates that one molecular species only is present and that the movement is anodic but only just detectable. The substances sediment in the ultracentrifuge as single entities but the form of the sedimentation boundaries indicates that the preparations are somewhat polydisperse. From the sedimentation and diffusion constants and the partial specific volume a molecular or 'particle' weight can be calculated for each material and is found to be quite large, something of the order 3×10^5 or larger. From the same data the frictional ratio f/fo for the substances can be computed and the values obtained indicate that the specific materials possess considerable structural asymmetry with axial ratios somewhat greater than 100. It is, however, uncertain to what extent hydration of the 'molecule' may be responsible for the high values found. The materials show no absorption peaks in the ultraviolet region (220–310 mμ). Some typical physical constants for the group substances are summarized in Table 3.

Chemical. Preparations of the group substances of both animal and human origin all appear to contain four sugars. Galactose and glucosamine were first detected by Freudenberg and Eichel (1934, 1935), fucose was isolated by Bray, Henry and Stacey (1946) as a methylated derivative, and the amino-sugar galactosamine was first identified by Aminoff and Morgan (1948). Eleven amino-acids—lysine, arginine, aspartic acid, glutamic acid, serine, threonine, glycine, alanine, valine, proline, leucine and/or isoleucine—have also been found as components of each of the highly purified group substances. Threonine is the amino-acid present in greatest amount whereas aromatic amino-acids appear to be absent.

TABLE 3. Physical Constants

Property	Blood-group substance			
	A	B	H	Le^a
Partial Specific Volume	0 635	0 600	0 636	0 643
Diffusion constant $D_{20} \times 10^7$ (1 0%)	1·6*	0 51†	1·21	1 37
Sedimentation constant $S_{20} \times 10^{13}$ (0 5%)	6 7	12 0	6 6	6 7
f/fo	3 2	5 6	4 2	3 8
Axial ratio	60	200	100	about 90
Particle weight	$2 6 \times 10^5$	$1 8 \times 10^6$	$3·2 \times 10^5$	$2 7 \times 10^5$

* 0 81%   † 0 2%

TABLE 4. Analytical Figures (Typical Values) for Preparations of the Human Blood-group Substances

	Rotation $[\alpha]_{5461}$	Nitrogen %	Acetyl %	Hexosamine %	Reduction %	Fucose %
A-substance	+15°	5 7	9 0	37	56	18
H-substance*	−30°	5 3	8 6	31	54	13
Le^a-substance	−40°	5 0	9 9	32	57	12
B-substance	0°	5 7	7 0	20	50	18

* For a material free from Le^b activity.

The results of these studies indicate the very interesting and important fact that in spite of the different blood group specificity of the A, B, H and Lewis Le^a substances, they all possess,

as far as we know at present, the same qualitative composition. Each contains L-fucose, D-galactose, D-glucosamine, D-galactosamine and eleven amino-acids, none of them aromatic amino-acids.

A set of typical analytical figures for each of the group substances is given in Table 4. The ratio of the amino-sugars in the materials has also been determined. For group A substances the glucosamine-galactosamine ratio varies in different preparations from 0·7 to 1·6, for group B and Lewis Le^a substances the ratio is nearer to 3 whereas for the H substances ratios from 2·6 to 12·7 have been obtained. The wide variation in the ratios found for H substances may be related to the very different serological characters of the H substances studied. It is of interest that the glucosamine content in most specimens is between 16 and 20 per cent. The galactosamine value is similar for A substances but falls to about half this value for B and Le^a substances whereas for some preparations of H substance as little as 2 per cent galactosamine has been found.

L-fucose is rapidly eliminated from the group substances by *N*-acetic acid at 100° and reaches a maximum value equivalent to about 80 per cent of the total fucose content which suggests that the fucose may be bound in the mucoid complexes in at least two ways.

Serological. During mild acid hydrolysis of the group A substance there is a steady loss of serological activity as measured by the capacity of the material to inhibit iso-agglutination but the property of inhibiting the haemolysis of sheep cells by rabbit anti-A serum, the so-called Forssman activity, is considerably enhanced.

The group substances show no or very little capacity to react with pneumococcus type XIV antibody but after hydrolysis there arises from each group mucoid, irrespective of its specificity within the ABO or Lewis systems, a structure which possesses a common or overlapping specificity with that shown by the specific polysaccharide of the pneumococcus type XIV. As a result of a careful study of these changes, Kabat, Baer, Bezer and Knaub (1948), van Vunakis and Kabat (1951) and Kabat (1952) have suggested that the fucose molecules in the

group substances project outward from the main chain and prevent or restrict the reactivity of these materials with pneumococcus type XIV antibody. Removal of the fucose by mild acid hydrolysis then uncovers structures reactive with the pneumococcus antibody. This pronounced cross-reactivity suggests that the N-acetyl-hexosamine-galactose chains make up major structures within the polysaccharide moieties of all the group substances and are similar in pattern to each other and to the N-acetyl-glucosamine-galactose structures of the pneumococcus type XIV specific polysaccharide.

Alkaline Hydrolysis of Group Substances

[illegible]

hydrate or the amino-acid-containing moieties or may be associated with the bonds which hold these two major components together to form the group specific macromolecule of 'native' blood group substance. It seems unlikely that peptide linkages are hydrolysed by mild alkali (pH 8–9) whereas it is known that certain carbohydrate structures are readily destroyed under these conditions. A detailed study has been made of the behaviour of the purified group substances on treatment with $BaCO_3$ (pH 8 5) at 100° and in an N_2 atmosphere. In order that the small molecules liberated during the early stages of the alkaline hydrolysis may not become decomposed during the extended treatment necessary to bring about complete degradation, the group substances, after short periods of heating, are dialysed and the indiffusible material again heated, the process of heating and dialysis being continued for many hours. After about 48 hr. at 100° the major part of the group substance has become diffusible.

There seems to be no preferential hydrolysis of a simple sugar, oligosaccharide, amino-acid or peptide. The hydrolysis proceeds steadily and apparently in very much the same manner throughout the disintegration of the group complex The total diffusible material arising from each group substance has been fractionated from water by ethanol and ether and separated into small

molecular entities by paper chromatography or by passage through charcoal-Celite columns. All the alcohol precipitable fractions contain all the components (fucose, galactose, amino-sugars and amino-acids). The fractions precipitated at high concentrations of alcohol and the addition of ether, probably have quite small molecular weights—less than twelve thousand—but some of these materials nevertheless have an overall composition closely similar to that of the original group substance.

It has been known for many years that mucins and mucoid substances give rise to an intense purple colour after heating with dilute alkali and the addition of p-dimethylamino-benzaldehyde (Ehrlich's reagent) in acid solution. A similar colour is given by the N-acetylaminosugars and it is now known that essential requirements for chromogen formation, that is, the capacity to react with Ehrlich's reagent, are that a free reducing group (CHO) must be adjacent to a ·CH.NH.CO.CH_3 group and that the hydroxyl group in position 4 of the N-acetylaminosugar must be unsubstituted. It is known that the O-glycosides of the N-acetylaminosugars do not give a positive reaction, as there is no free CHO group, and, of course, most O-glycosides are stable to mild alkali. However, the group substances after alkali treatment give a greater amount of colour, in terms of N-acetylglucosamine, than can arise from reducing N-acetylaminosugar end units originally present in the undegraded mucoid complex. One must conclude, therefore, that treatment of these materials with dilute alkali leads to the hydrolysis of the glycoside linkages in an alkali-labile amino-sugar containing chain (Knox and Morgan, 1954). A series of oligosaccharides which show in collidine R_F values from 0·05 to 0·78, and which are composed largely of N-acetylaminosugar residues has been isolated from the diffusible material obtained from the group substances after heating at pH 8·5 and since both amino-sugars are present in the units liberated it seems that chains rich in alkali-labile amino-sugar are important structural elements in the native group substances. That only a part of the total N-acetylhexosamine present in each group substance is involved

in forming the chromogen structure suggests strongly that these complex materials have their hexosamine molecules linked in two different forms, most probably as components of two different carbohydrate chains, one of which is alkali-labile and gives the chromogen, the other alkali-stable and showing no tendency to break down and be converted into chromogenic structures.

The largest of the diffusible substances derived from A and H materials by treatment at pH 8·5 are serologically active. The small amount of indiffusible material remaining after extensive hydrolysis, however, is serologically inactive.

Oxidation of Group Substances with Periodate

Oxidation of the group substances with periodate offers an [illegible]

age in substances containing either two adjacent unsubstituted hydroxyl groups or an unsubstituted hydroxyl group adjacent to a primary or secondary amino group. Oxidation reaches completion in a few hours at pH 5 (Aminoff and Morgan, 1951) and in spite of the fact that oxidation is extensive the group substances remain indiffusible through a cellophan membrane and it appears that there is no extensive breakdown of the macromolecular structure to units of less than about 12,000 molecular weight, as occurs after treatment with dilute alkali. Chromatographic analysis of the acid hydrolysis products of the oxidized materials shows that with each group substance fucose and galactose are completely destroyed together with about a quarter of the amino-sugar units. There is, however, no destruction of the amino-acids At pH 3·0, about 1 μg. only of formaldehyde is produced from 1 mg of the group substances which suggests that not more than one —CHOH—CH_2OH grouping is present per 130 'hexose' residues in the group substances and that under conditions where 'overoxidation' is reduced to a minimum, the amount of HCHO produced is probably not sufficient to be of definite structural significance.

Hydrolysis of the group substances with acetic acid rapidly

splits off fucose. The oxidation at pH 3 of the indiffusible hydrolysis products would therefore reveal if furanose-sugars were being 'masked' by fucose or other acid-labile components. The results of such an experiment suggest that structures containing free OH groups at C atoms 5 and 6 are formed during the early part of the hydrolysis but that after about 16 hours at 100°, when almost the whole of the acid-labile fucose is liberated, the products give less and less formaldehyde, suggesting that the formaldehydogenic structures are acid-labile. There is some indication that with certain group B substances only very short periods (2 hr.) of hydrolysis are necessary before maximum liberation of formaldehyde occurs on subsequent oxidation.

THE OXIDATION OF THE GROUP SUBSTANCES WITH HYPOIODOUS ACID

Under conditions which practically exclude 'overoxidation', that is at 2° and pH 9·5, an initial rapid oxidation of the group substances with hypoiodous acid is complete in about 6 hours. Presumably only reducing CHO end-groups are oxidized to carboxyl groups, for the Somogyi copper reduction method gives, as expected, about one-third of this value. In general the reducing power of the group substances, determined with hypoiodous acid and expressed as glucose, is between 2 and 3 per cent and the 'unit' weights are thus found to be about 6,000. If the particle weight is taken as approximately 300,000 then there are about 50 carbohydrate chains each possessing a reducing group in each macromolecule of blood group substance. The occurrence, within a macromolecular structure of this size, of so many carbohydrate chains with reducing end-groups is unusual and is probably due to the structural integration brought about by the amino-acid-containing moiety. It is possible, however, that even at 0° and pH 9·5 there are glycoside linkages labile enough to be hydrolysed, perhaps N-glycosides.

The oxidized group substances were recovered, after thorough dialysis, in yields of about 95 per cent. Examination by electrophoresis and ultracentrifugation at pH 8·0 showed that these oxidized substances were substantially homogeneous and that no gross molecular degradation had taken place. The

analytical figures show that for group A substances galactosamine is the amino-sugar involved in oxidation, but for the other group materials the results were not so simple to interpret and sometimes both amino-sugars and/or galactose and fucose seem to be involved in oxidation. After oxidation with hypoiodite, the A, B and H substances retain their serological activity and it appears that the reducing end-groups are not essential for immunological specificity.

Enzymic Decomposition of the Group Specific Substances

Another approach to the determination of the structure of the specific blood group substances is that which involves the use of enzymes. Pepsin, trypsin and papain have no action so far as we are aware and there is no loss in specific serological properties of the group materials after treatment with these enzymes.

Many years ago Freudenberg showed that the group substances obtained from human urine were destroyed by snail enzyme and N-acetylglucosamine was detected in the products of enzymic hydrolysis. Howe and Kabat (1953) have taken up again the use of snail juice as a hydrolytic agent Their results are difficult to interpret because the enzymic hydrolysis is very slow and complex and takes some weeks to reach equilibrium. There is no evidence that the enzyme preparation does not contain many carbohydrases and it is impossible at the moment to know the order or nature of the chemical bonds split during the destruction of immunological specificity.

The same criticism applied to similar studies using culture filtrates from *Cl. welchii* (Morgan, 1946; Stack and Morgan, 1949; Crumpton and Morgan, 1950), but from this source [illegible]

enzyme in the protozoan flagellate *Trichomonas foetus* which brings about the serological inactivation of the human H substance, with extensive release of fucose but only a very small amount of N-acetylhexosamine and no galactose, will probably allow the enzymic approach to the problem of mucoid structure

to be extended. The action of this enzyme preparation and that obtained from *Cl. welchii* can be specifically inhibited by simple sugars of known structure. It has been found that the enzyme which inactivates the A substance is inhibited in its action on that substance by N-acetylgalactosamine but not by other sugars. The group B substance is decomposed by an enzyme which is inhibited by D-galactose or simple sugars which contain galactose, but not by other sugars. Finally the H substance is protected from enzymic attack in the presence of L-fucose and D-galactosamine but not by galactose or N-acetylgalactosamine. The enzymes inhibited by these sugars are presumably the ones which produce the initial change that brings about loss of immunological specificity and the results enable a tentative conclusion to be drawn as to the specific activity of the enzymes concerned. It seems probable that the sugars which bring about the inhibition observed are those which play an important rôle in determining the characteristic serological specificity of the blood group substances (Watkins and Morgan, 1955).

CONCLUSIONS

The isolation and characterization of the blood group substances has been subjected to careful study over the past few years and it is now generally believed that the specific serological characters within the ABO and Lewis groups are due to well-defined chemical entities known as mucopolysaccharides, or more precisely as polysaccharide-amino-acid complexes. The A, B, H and Le^a substances show many chemical properties in common and the results of qualitative analysis indicate that, irrespective of their group specificity, they are all built up from the same chemical units—L-fucose, D-galactose, N-acetylglucosamine and N-acetylgalactosamine and eleven amino-acids. There is a marked difference in the glucosamine-galactosamine ratio in the different group substances, the ratio being close to 1·5 for A substances but three or higher for H preparations. The B substances contain less amino-sugar than the A, Le^a or H substances but the glucosamine content of each material is found to be approximately the same.

The group materials belong to a special type of polymolecular aggregate which possesses a very large particle weight (3×10^5 or 1×10^5) and which is extremely unstable in the presence of mild alkali.

Studies on the group substances which have involved (*a*) oxidation with periodate and (*b*) hypoiodous acid, (*c*) enzymic decomposition, (*d*) acid and enzymic degradation to form a material with new serological specificity, (*e*) hydrolysis with weak acid and treatment (*f*) with mild alkali, have given results which allow a tentative and common structure to be pro-

dilute alkali and composed largely or entirely of N-acetylhexosamine units, are stages in the degradation of the alkali-labile N-acetylhexosamine chains. It is to be noted that the amino-sugars are present in an alkali-labile condition, which is certainly not so for the amino-sugars occurring in the majority of other macromolecules, such as in chitin, in most bacterial antigens or in the acid mucopolysaccharides of tissues. The component amino-sugars could be linked as alkali-labile 1 : 3 pyranose units or as 1 5 or 1 : 6 linked furanose structures. But as yet we have no direct evidence for the presence of furanose structures.

The second type of carbohydrate chain is probably composed in part of galactose and N-acetylglucosamine units, and it is this carbohydrate structure which gives rise to the cross reacting pneumococcal type XIV activity following treatment of the group substances with weak acid This chain may well be substituted in some of its free hydroxyl groups by L-fucose, which is readily removed by hydrolysis with mild acid but which is firmly attached glycosidically to the main chain by an alkali-stable linkage. The identification by Kuhn and Kirschenlor (1954) of 4-*o*-β-D-galactosido-N-acetyl-D-glucosamine as a component of a mixed preparation of human blood group mucoids derived from meconium and the relative stability of the pneumococcus type XIV specific polysaccharide to alkali,

suggests that the N-acetylglucosamine and galactose residues in the group substances are joined by an alkali-stable 1 : 4 glycosidic linkage.

It seems therefore that there are at least two kinds of carbohydrate chains present in each group substance, an alkali-labile N-acetylhexosamine-rich chain and an alkali-stable chain which contains galactose and N-acetylglucosamine units.

The group mucoids possess an amino-acid-containing moiety as an integral part of their structure and so far a fully serologically active material which is devoid of amino-acids has not been obtained. It seems that the peptide moiety is held together by carbohydrate chains which act as bridges and bring about the formation of a macromolecular structure. The bonds which join the carbohydrate and peptide chains together are alkali-labile and their hydrolysis causes complete disruption of the group complexes.

A part of an hypothetical structure which will account for many of the observed reactions of the group substances is given in Figure 2. The peptides are indicated in the conventional manner and the reducing or potentially reducing end-groups of the carbohydrate chains by the sign →. The linkages sensitive to alkali and readily hydrolysed are shown with a vertical dotted line. Hydrolysis of these alkali-labile bonds would lead to a complete break up of the group substance.

Carbohydrate chain A is envisaged as joined through its potential reducing group to the peptide chain by an O-glycosidic bond which would be stable to mild alkali. The carbohydrate would, however, be split from the peptide chain at its non-reducing end, where it is joined to the amino-acid chain by an alkali-labile ester linkage. The carbohydrate would be neither degraded nor liberated.

Carbohydrate chain B on the other hand is considered as joined to the peptide by an alkali-labile N-glycosidic linkage at the reducing end and an alkali-stable ether linkage at the non-reducing end. On treatment with alkali the carbohydrate chain B would be readily degraded owing to the formation of a reducing end-group. It is possible that an alkali-labile linkage joins a carbohydrate chain at the reducing end and at the non-

reducing end. The carbohydrate chain C is placed in this hypothetical structure because such a chain composed of N-acetylaminosugar units, attached to the peptide through an

```
   |                                                   |
   NH                                                  NH
   |                                                   |
   CH—R—COO¦——————A——————→        O—R—CH
   |                                                   |
   CO                                                  CO
   |                                                   |
   NH                                                  NH
   |                                                   |
—R—CH                                                  CH—R—
   |                                                   |
   CO                                                  CO
   |                                                   |
   NH                                                  NH
   |                                                   |
   CH—R—O ——————————B——————→¦NH—R—CH
   |                                                   |
   CO                                                  CO
   |                                                   |
   NH                                                  NH
   |                                                   |
   CH—R—COO¦——————C——————→                   CH—R—
   |                                                   |
   CO                                                  CO
   |                                                   |
   NH                                                  NH
   |                                                   |
   CH—R—O ←——————A————————¦ OOC—R—CH
   |                                                   |
   CO                                                  CO
   |                                                   |
   NH                                                  NH
   |                                                   |
   CH—R—NH¦←——————B————————— O—R—CH
   |                                                   |
   CO                                                  CO
   |                                                   |
   NH                                                  NH
   |                                                   |
—R—CH            ←——————C—————————¦ OOC—R—CH
   |                                                   |
   CO                                                  CO
   \                                                   )
```

FIG. 2. A hypothetical structure for the specific blood group mucopolysaccharides.

alkali-labile ester linkage and itself possessing alkali-labile glycosidic linkages and a reducing end-group, would probably give rise to a series of N-acetylhexosamine oligosaccharides as found experimentally.

The blood group substances in man are major constituents of the normal mucilaginous secretions—saliva, gastric and intestinal mucin, etc.—and as such are almost certainly essential for the normal working of the body. It seems that the characteristic immunological specificity with which the various components of the mucin are endowed is of no importance in terms of physiological function, mucoids with A, B, H or Le^a specificity are apparently equally efficient in performing the functions demanded of mucin in the body. However, the fact that the group substances possess a characteristic immunological specificity which is gene controlled and which sharply differentiates the one mucoid from the other establishes these materials as 'markers' of outstanding value in the field of human biochemical genetics. The determination of the precise differences in closely related gene products is of outstanding importance, for our comprehension of gene action cannot be complete until the fundamental chemical changes involved are fully understood.

One of the tasks of the immunochemist in this field is to isolate these important group specific materials free from similar substances which possess other serological characters, to establish their chemical nature and to determine the ways in which they differ from each other and, if possible, to associate a given blood group specificity with a known chemical structure.

The first stages in this task have been accomplished—the specific materials have been isolated and characterized. We should like more elegant methods of isolation and more perfect methods for characterization but I have no doubt that these will come in the near future. The ways in which these different gene products differ chemically from each other are being slowly revealed. The essential chemical structure which is responsible for the blood group specificity, however, remains unknown to us. I think it is possible to see just where we shall get this information. Much remains to be done in this important field

of study but I hope you will agree that a useful beginning has been made.

REFERENCES

AMINOFF, D. and MORGAN, W. T. J (1948). *Nature, Lond.* **162**, 579

AMINOFF, D. and MORGAN, W. T. J. (1951). *Biochem. J.* **48**, 74

AMINOFF, D, MORGAN, W T. J. and WATKINS, W M (1950). *Biochem J.* **46**, 426.

ANNISON, E. F. and MORGAN, W. T. J. (1952a). *Biochem. J.* **50**, 460.

ANNISON, E F. and MORGAN, W. T. J (1952b) *Biochem. J.* **52**, 247.

BHENDE, Y. M., DESHPANDE, C. K, BHATIA, H M, SANGER, R, RACE, R. R, MORGAN, W. T. J. and WATKINS, W. M (1952) *Lancet*, **1**, 903.

BRAY, H. G, HENRY, H and STACEY, M (1946) *Biochem. J* **40**, 124

BUCHANAN, D. J. and RAPOPORT, S (1951). *Proc. Soc exp Biol., N.Y.* **77**, 114

CASPARY, E. A. (1954). *Biochem J.* **57**, 295.

CRUMPTON, M. J. and MORGAN, W. T. J. (1953). *Biochem. J.* **54**, xxxii.

FREUDENBERG, K. and EICHEL, H. (1934). *Liebigs Ann.* **510**, 240.

FREUDENBERG, K. and EICHEL, H. (1935) *Liebigs Ann* **518**, 97

GIBBONS, R. A, MORGAN, W. T J. and GIBBONS, M (1955). *Biochem. J.* **60**, 428.

HOWE, C. and KABAT, E. A (1953). *J. Amer. chem. Soc* **75**, 5542

KABAT, E A. (1949). *Bact. Rev.* **13**, 189

KABAT, E A. (1952) *Harvey Lectures*, p 252, Springfield, Ill

KABAT, E. A., BAER, H., BEZER, A E and KNAUB, V (1948). *J. exp Med* **88**, 43.

KABAT, E A. and BEZER, A. E. (1945) *J. exp. Med* **82**, 207.

[illegible]

[illegible] 60

MORGAN, W T. J. (1947) *Experientia*, **3**, 257

[illegible]

STAC[illegible] *J exp Path* **30**, 470.

VAN[illegible] *er chem. Soc.* **73**, 2977.

WATI[illegible]

WAT[illegible] *Brit J exp. Path* **35**, 181

WATKINS W M and MORGAN, W T J (1955) *Nature, Lond* **175**, 676

VII

The Plasma Proteins

R. A. KEKWICK

Introduction

In view of the wide range of information which exists about the plasma proteins, it is only by a rigorous selection, limiting this rather general account to certain aspects of the subject, that anything useful can be achieved in a short survey.

Accordingly this discussion will be confined chiefly to the human plasma proteins. Some historical aspects of the characterization of the plasma proteins, from a physico-chemical point of view, will be followed by illustrations of the kind of alterations which occur in the plasma protein distribution in several disease conditions as revealed for the most part by electrophoresis studies. Finally the impact of the bulk fractionation of the plasma proteins on our knowledge of the individual constituents of plasma, and some implications of this knowledge, will be discussed.

Characterization of the Plasma Proteins

There are two main categories of information concerned in the characterization and differentiation of the various proteins of plasma. First, general physico-chemical data, especially such as may be obtained with the ultracentrifuge and electrophoresis apparatus, and secondly those relating to a specific biological activity susceptible to quantitative measurement, such as an enzymic or immunological property. It is by the interplay between these two categories of information that many advances have been made during the last few years

Salting out. The plasma proteins were first differentiated in terms of their solubilities in aqueous salt solutions. By this means the albumins and globulins were distinguished the latter being sub-divided into euglobulins and pseudoglobulins. At pH values close to 7 0 the albumins were defined as the protein fraction soluble in 0 50 saturated $(NH_4)_2SO_4$, the globulins as the fraction insoluble under these conditions. When the globulin precipitate obtained by 0·5 saturation with $(NH_4)_2SO_4$ is redissolved in water and the salt contaminating the precipitate removed by dialysis, an insoluble subfraction is obtained which is defined as euglobulin, the soluble fraction being pseudoglobulin. Subsequently these fractions were partially redefined in terms of salting out procedures alone, the euglobulin fraction being that insoluble in 0·33 saturated $(NH_4)_2SO_4$ and the pseudoglobulin the fraction precipitating between 0 33 and 0 50 saturation with $(NH_4)_2SO_4$.

The introduction of sodium sulphate as a precipitating agent by Howe (1925) facilitated the rapid estimation of these protein fractions by Kjeldahl nitrogen estimation. Much information has accumulated about the quantitative variations occurring during many disease processes, and has proved to be of considerable value in the selection of material for examination by more recently devised methods.

Ultracentrifuge Measurements. Studies with the ultracentrifuge (McFarlane, 1935; Pedersen, 1945) revealed that the major portion of the plasma proteins could be accounted for mainly in terms of two species of different molecular size, with sedimentation coefficients of about 4 5 and 7 0 S; these corresponded approximately to the albumin and globulin fractions Fibrinogen does not resolve from these components but probably sediments in the 7·0 S peak.

In addition, other size-species of proteins occur in the normal plasma of many animals, in man there is notably one with a sedimentation coefficient of about 20 S with which iso-agglutinin and possibly some other agglutinin activity (Typhoid O antibodies) are associated This component accounts for a relatively small proportion of the total plasma proteins.

It is important to realize that there is a lower limit of con-

centration of protein below which the presence of constituents can be detected neither in the ultracentrifuge nor in the electrophoresis apparatus; measurements with both of these instruments depend on the same optical method. In consequence, some components may not be detected until they have been separated from the main bulk of the plasma proteins and obtained in a concentrate by an independent fractionation procedure.

There is another characteristic of the sedimentation behaviour of human plasma, which derives from the presence of lipoprotein. Lipoproteins have a density in solution in the range 0·99–1·05 whereas the value for simple proteins is mostly about 1·3. Consequently, when plasma is dialysed against a salt medium whose density is greater than 1·05 the lipoprotein components will not sediment in the ultracentrifuge, but will move towards the centre of rotation. Such components may be characterized by 'flotation' (S_F) rather than sedimentation coefficients. In general, the apparent sedimentation velocity of these constituents is much more sensitive to slight density changes in the medium than that of ordinary proteins, a phenomenon first observed by Macfarlane (1935) and more extensively studied by Pedersen (1945). Recently, Gofman and his associates (Lindgren, Elliot and Gofman, 1951) have concentrated lipoprotein fractions from human plasma by flotation in a preparative ultracentrifuge and examined the concentrates in the analytical ultracentrifuge. They demonstrated the existence of lipoprotein components with a wide range of flotation coefficients from S_F 2 to 40, and suggest that the amount of lipoprotein components with S_F values of 10–20 present in plasma shows some correlation with the incidence of atherosclerosis.

Electrophoresis Measurements. The study of the plasma proteins by moving boundary electrophoresis in the apparatus devised by Tiselius (1937a) has probably contributed more to our knowledge than study in the ultracentrifuge.

In the ultracentrifuge the behaviour of proteins is dependent primarily on their molecular size and shape, and secondarily on their density. Density certainly reflects differences in chemical

constitution but only those of a gross nature as in the lipoproteins, where as much as 70 per cent by weight of the molecule may be lipoid. The electrophoretic behaviour is much more closely related to the amino-acid constitution of the proteins, since under specified conditions the mobility is a function of the number of ionized groups on the molecule. Even so, excluding for the moment specific ion binding effects, only the trivalent basic and acidic amino-acids contribute directly to the charge on the molecule, the bulk of which is constituted of mono-amino mono-carboxylic acids whose ionizing groups, being masked in peptide bonds, can have merely an indirect influence.

With the electrophoresis apparatus it was shown by Tiselius (1937b) that at pH values close to 8·0, the serum proteins could be differentiated electrochemically in decreasing order of their electrophoretic mobility into albumin and three globulins, alpha, beta, and gamma. Shortly after this Stenhagen (1938) demonstrated that in plasma, fibrinogen migrated as a separate component with a mobility between that of beta and gamma globulin. In later studies, notably by Longsworth (1942), the presence in human plasma of two alpha globulins, $alpha_1$ and $alpha_2$, was established; in some animal species there are further qualitative differences in the normal plasma indicating that more than one electrophoretically resolvable component may occur in the beta and gamma globulins, depending on the species.

The quantitative protein constitution of normal human plasma as found by electrophoretic analysis is expressed usually in terms of the proportional contribution to the refractive index made by each of the proteins present. More precisely the percentage of the total protein attributable to any one component is determined from the peak areas of the electrophoresis schlieren diagrams. The data may be transformed into grammes of protein/100 ml plasma by using the specific refractive increment value appropriate to each component but this is done only when special reasons make it desirable.

Electrophoretic examinations by many workers in various countries indicate that the distribution of the proteins in normal

human plasma is remarkably constant. This is in rather marked contrast with animals such as the horse, cow, pig and sheep where there is much greater variability.

It is worth mentioning that electrophoretic examination of the protein fractions obtained from plasma by salt precipitation, which were mentioned earlier, reveals that they are all mixtures of the various electrophoretic components. Nevertheless, broadly speaking, the euglobulins contain the bulk of the γ-globulin with the less soluble portion of the β-globulins; the pseudoglobulins consist of β-globulin and the less soluble α-globulins; the albumin fraction separated by these procedures is always contaminated with α-globulin and traces of β-globulin.

The electrophoretic mobilities of the plasma protein components under specified conditions of pH and buffer ion concentration are used to characterize the proteins. However, to varying extents, the components are electrochemically heterogeneous and each would be represented more adequately by a range of mobility values rather than a single value. For this and other reasons each component should more properly be regarded as a family of proteins possessing rather similar physicochemical characteristics. Moreover, each component must probably comprise many proteins of diverse biological function, since the number of specific biological activities that have been demonstrated in plasma is far in excess of the seven or eight components that are electrophoretically distinguishable.

The Plasma Proteins in Disease

The changes occurring in the plasma proteins during disease processes have been excellently reviewed by Gutman (1948) and at the outset it may be useful to quote his opinion on the value of electrophoretic analysis. 'When first introduced it was hoped by some that electrophoretic analysis would provide characteristic and specific spectra in different diseases and be of great diagnostic value. It is now evident that the diagrams obtained in most disorders are not specific for any one disease but follow definite common patterns.' This, of course, does not signify that the information obtained is of little or no value.

There are in general several ways in which the plasma protein distribution might alter during disease. There may be a relative or absolute change in the amount of any of the normal components, either an increase or decrease, or there may be qualitative changes caused by the appearance of apparently abnormal proteins. In the ultimate analysis the 'abnormal' protein might be difficult to distinguish from large amounts of normal constituents ordinarily present in small traces. Rather than attempting an exhaustive survey, a few conditions in which rather marked changes occur will be used as illustrations.

Multiple myelomatosis. In this condition there is usually a hyperproteinemia and the most frequent electrophoretic finding is an enormous increase in a rather sharply defined γ-globulin, and less frequently a similar increase in a β-globulin component. In the ultracentrifuge the 'gamma myeloma' mostly shows an increase in the 7 S component though large amounts of faster-sedimenting components occasionally appear. In the 'beta myeloma' the ultracentrifuge diagram may be extremely complex. In some instances an increase in α-globulin has been recorded and in others the plasma has apparently remained quite normal.

Cirrhosis. In cirrhosis, though the γ-globulin is increased in amount, it is qualitatively different from the γ-globulin in myelomatosis, in that it displays a greater degree of heterogeneity. In addition, the amount of albumin is decreased though the total plasma protein tends to remain within the normal range.

Hypogammaglobulinaemia. Recently several individuals have been found in whose plasma γ-globulin is almost completely lacking, and who suffer from recurrent infections of various kinds On the basis of the electrophoretic findings this condition has been designated agammaglobulinaemia, though traces of γ-globulin may be present, but below the level of concentration detectable by electrophoresis.

Nephrosis. In this condition, characterized by proteinuria, the occurrence of oedema and a high plasma cholesterol, the electrophoretic diagram of the plasma reveals an extreme reduction in the amount of albumin, (0 5–0 9 g /100 ml.) and

excessively large α- and β-globulin peaks. The urinary protein distribution simulates that of normal plasma.

Simple extraction of the plasma with ether at room temperature reduces the amount of the β-globulin component dramatically, indicating that the large peak is predominantly due to the association of the increased plasma lipids with the β-globulin. This association of lipid and β-globulin is of a much less stable nature than that occurring in the normal β-lipoproteins.

Miscellaneous. An increase in α-globulins constitutes a rather striking and consistent manifestation in acute febrile diseases.

In malnutrition, it has been believed that the occurrence of oedema is definitely linked with hypoalbuminaemia. However, an extensive recent study (McCance *et al.*, 1951) has shown that though the total plasma protein falls the electrophoretic analysis changes only slightly, in the direction of a reduced proportion of albumin, and this is offset by a slight increase in α-globulin. However, in cases with almost identical plasma protein content and distribution, one may be oedematous and another not, suggesting that some factor affecting capillary permeability must be involved in addition to the lowered osmotic pressure of the plasma.

The interpretation of the significance of the changes in the plasma proteins in disease is in most instances very obscure. The plasma protein picture is the result of the dynamic balance between production of plasma proteins and their breakdown or loss. Until more is known of the origin and normal turnover rate of the plasma proteins, and the effect of specific disease processes on the turnover rate of individual proteins, a subject which is now being actively studied with radio-isotope techniques, it will remain difficult to evalute fully the data which are already at our disposal.

BULK FRACTIONATION OF NORMAL HUMAN PLASMA AND THE CHARACTERISTICS OF SOME NORMAL HUMAN PLASMA PROTEINS

In attempting further to elucidate the changes in human plasma proteins in pathological conditions, it is necessary to have as much information as possible about the characteristics

and functions of the normal proteins, in order to assess quantitatively variations in their amounts and biological effects.

Many facts relevant to this problem were elicited as a result of the clinical demand for large quantities of citrated blood for the transfusion of casualties in the 1939–45 war; because for the first time normal human plasma consequently became available in amounts that made it essential to devise and operate bulk processing schemes in order to salvage as much as possible of such biologically important material. The bulk fractionation of the plasma proteins was one of the ways adopted for dealing with this problem, and in addition to providing protein fractions of great value clinically this has been instrumental in advancing our knowledge of the detailed properties of the plasma proteins, especially of those normally present in small amounts but with highly specific characteristics.

The reintroduction by Cohn and his colleagues (1946) of water miscible solvents as protein precipitants at temperatures in the range 0° to – 10° C. under controlled conditions of pH, ionic strength, ion composition of the medium and protein concentration, provided much more sensitive fractionating conditions than those previously used.

The fractionation methods which have been devised have aimed initially at the separation of the plasma proteins into a limited number of main fractions corresponding as closely as possible with the individual electrophoretic components. These main fractions are then subfractionated more particularly with respect to specific biological and biochemical characteristics, with a view to recovering in a highly purified condition as much as possible of such constituents It is beyond the scope of this review to consider the finer details of these subfractionation procedures However, it is of value to see how various biologically active proteins are distributed among the main fractions, and to give some account of their physico-chemical characteristics in those instances where the preparations obtained have been sufficiently pure to justify such measurements being made.

Serum albumin. Serum albumin is probably the least complex of the plasma proteins both biologically and physico-chemically. Its main function appears to be osmotic regulation; although

it constitutes only about 55 per cent of the total plasma proteins it is responsible for about 80 per cent of the osmotic effect of plasma mainly because its molecular weight, 65,000, is low in comparison with the other major constituents. It probably has also an important function in relation to its capacity to bind calcium and perhaps in the transport of fatty acids. That it is chemically heterogeneous has been shown by Hughes (1950), who from the interaction of serum albumin with mercury found that about 60 per cent of the molecules had one free –SH group and the remainder none.

γ-globulins. The association of antibody activity with the globulin fraction of plasma was established relatively early using salting out procedures. In 1939 Tiselius and Kabat demonstrated that in the hyperimmune rabbit, antibody was associated with the γ-globulins. This is not invariably the case since there are marked differences from species to species, and antibodies may be present in other electrophoretic components.

In man most of the antibodies occur in the γ-globulin as originally defined. However, examination of the bulk fractions by Enders (1944) indicated a spread over towards the β-globulin region. Deutsch, Alberty and Gosting (1946) showed that a component migrating between the γ- and β-globulins carries the iso-agglutinin activity and typhoid 'O' agglutinins. This fraction contains a high proportion of a component with a sedimentation coefficient of about 20 S with which the agglutinins are associated. They proposed that this component should be designated γ_1-globulin and the 'classical' gamma globulin, γ_2

Leaving out of consideration this complication, the 'classical' γ-globulin is electrochemically very heterogeneous and contains components whose isoelectric points cover a range from pH 6·7 to 7·5. In the ultracentrifuge, preparations of the protein appear to be relatively homogeneous, with a molecular weight of 160,000.

The successful clinical use of γ-globulin in the attenuation of measles needs no emphasis.

Fibrinogen. With the fibrinogen fraction most of the antihaemophilic factor is precipitated. Like many of the proteins concerned in the clotting mechanism, the latter appears to be very

labile and its recovery from human plasma has so far been very poor.

The molecular weight of human fibrinogen has been redetermined recently on preparations of high purity, free from plasminogen and plasmin; a value of 340,000 was obtained. There is some evidence that the fibrinogen molecule may dissociate in dilute solution (0·15 g./100 ml.) into units of the order of 100,000 molecular weight (Caspary and Kekwick, 1954).

A globulin which is extremely insoluble at low temperatures also separates with the fibrinogen and may be related to the so-called cryoglobulins sometimes found in pathological plasmas.

The α- and β-globulins. The fact that most of the plasma lipids are carried with these components was established by Blix, Tiselius and Svennson (1941) and also that they contain much protein-bound carbohydrate.

There is little doubt that these groups are at once the most complex, most difficult to fractionate and probably most versatile of the plasma electrophoretic components in that they embrace a wide variety of biological activities. Here are found prothrombin, antithrombin, 'Christmas factor', copper and iron binding proteins, enzymes such as pseudocholinesterase and components of complement.

The separation and purification of many of these is at present rudimentary, and there are many constituents whose function has yet to be elucidated.

Conclusion

The range of the physico-chemical properties of those protein constituents of plasma which have been prepared in a relatively high degree of purity is much wider than might reasonably have been anticipated from the earlier ultracentrif...

phoretic components, more particularly by the α- and β-globulins, again emphasizes the limits of discrimination to which the electrophoretic data on whole pathological plasmas are subject

The theoretical aspects of ultracentrifuge and electrophoresis measurements have been developed considerably in recent

years, and it is possible to compare quantitatively, in terms of the spreading of the boundaries of protein preparations showing only a single component, the degree of physico-chemical homogeneity. For such constituents as enzymes and antibodies an independent assessment of purity is available in terms of the activity per unit mass of protein.

In conclusion I would like to draw attention to a method newly devised by Grabar and Williams (1953), which combines electrophoretic migration with an immunological method of detection. With this, trace protein contaminants, present in preparations classed as apparently homogeneous by other procedures, may be detected and distinguished qualitatively, though at present the method does not furnish a quantitative measure of these contaminants. Results obtained with this method will undoubtedly be of great value and lead to further advances in our knowledge of the plasma and other proteins.

REFERENCES

BLIX, G , TISELIUS, A and SVENNSON, H. (1941). *J. biol Chem.* **137**, 485.

CASPARY, E. A. and KEKWICK, R. A. (1954) *Biochem. J.* **56**, xxxv.

COHN, E. J., STRONG, L. E , HUGHES, W L , MULFORD, D. J., ASHWORTH, J. W , MELIN, M. and TAYLOR, H. L (1946). *J. amer. Chem Soc.* **68**, 459.

DEUTSCH, H. F., ALBERTY, R. A. and GOSTING, J (1946). *J. biol. Chem.* **165**, 21

ENDERS, J. F (1944) *J. clin Invest.* **23**, 510

GRABAR, P and WILLIAMS, C A. (1953). *Biochem biophys. Acta*, **10**, 193.

GUTMAN, A B. (1948) *Advances in Protein Chemistry*, **4**, 155.

HOWE, P. E (1925). *Physiol. Rev* **5**, 439

HUGHES, W L. (1950) *Cold Spr. Harb Symp. quant. Biol.* **14**, 79

LINDGREN, F. T., ELLIOT, H. A and GOFMAN, J. W. (1951). *J phys. Chem.* **55**, 80.

LONGSWORTH, L G. (1942) *Chem Rev.* **30**, 323.

MCCANCE, R. A. and others (1951). 'Studies of undernutrition, Wuppertal, 1946–9'. Med Res Council Spec. Report, Series No. 275

[illegible]

[illegible] *and serum fractions.*

[illegible]

TISELIUS, A. (1937b). *Biochem. J* **31**, 1464

TISELIUS, A. and KABAT, E A. (1939) *J. exp. Med* **69**, 119

VIII

Laboratory and Clinical Findings in Hypersensitivity

JOHN R. SQUIRE

FOUR years ago, in this same series of lectures, I was able to review some of the different kinds of protein sensitization (Squire, 1952). Since then, considerable progress has been made, both in understanding the body mechanisms involved in the various types of reaction, and also in applying physico-chemical and immunological techniques to the materials provoking hypersensitivity and to the sera of affected patients or animals.

TYPES OF HYPERSENSITIVITY

Various forms of classification of hypersensitivity reaction may be adopted depending on the purpose in mind. One might, for example, group together conditions affecting a specific tissue, such as the skin. More fundamentally, one might separate reactions suppressed or unaffected by particular drugs, such as anti-histamines. From the immunological view-point at the present time, it seems best to distinguish two classes of reaction, those in which an antibody can be demonstrated circulating in the plasma of hypersensitive individuals, and those in which no such demonstration has been achieved. For example, one may instance:

Group I. Antibody demonstrable in plasma, reactions usually of rapid type.

(*a*) Asthma—hay-fever, urticaria—group.

(*b*) Certain forms of drug sensitivity, e g. Sedormid purpura.

Group II. No antibody found in plasma, reactions usually of delayed type.

(*a*) Tuberculin-trichophytin group.
(*b*) Contact dermatitis.

Such a classification needs careful and repeated scrutiny to decide whether the separation and implied interrelations are fundamental or superficial. The demonstration of antibody in Group I (*a*) conditions is usually only achieved by the technique of passive transfer (Prausnitz-Kustner reaction) of hypersensitivity to a normal individual, though more will be said later of attempts to devise an *in vitro* test for this form of antibody. Group I (*b*), Sedormid purpura, is fortunately not a common condition. But the special contribution of Ackroyd (1949, 1954) in studying Sedormid purpura has been to show clearly that the plasma of affected patients contains an antibody, demonstrable by admixture with normal platelets and the drug *in vitro*. Under suitable conditions, platelet agglutination, lysis and fixation of complement ensue. This form of test may eventually form a model for the *in vitro* study of plasma antibodies in hypersensitivity.

The validity of separating Group I from Group II conditions seems to have been strengthened in recent years by the demonstration that hypersensitivity of the latter kind can be transferred, not by plasma, but by suspensions of mononuclear cells from the affected individual. This may imply a fundamentally different mechanism, or merely that the kind of antibody postulated as being concerned is very closely linked to body cells. Further work is required to answer this problem with certainty. Meanwhile, it is noteworthy not only that the specific hypersensitivity is transferable by cell suspensions, but also that the very nature of the reaction involves the participation of large numbers of cells which migrate into and multiply in the region where the tuberculin, for example, has been introduced. The possible relations and differences between Group I and Group II conditions have been recently discussed in full by Gell (1953). The currently accepted classification into Group I and Group II disorders will need radical reconsideration if the results of Cole and Favour (1955), published after the preparation of this lecture, are confirmed. Sera from guinea-pigs sensitized with tubercle bacilli have been fractionated by chemical

procedures. An immediate wheal-type reactivity to tuberculo-polysaccharide was passively transferred to normal animals with a γ-globulin fraction. A delayed, probably tuberculin-type hypersensitivity to tuberculoprotein was transferred with a fraction IV-10 rich in α-globulin. Admixtures of these two fractions transferred the immediate type hypersensitivity only.

Lately, special attention has been paid to the cellular reaction in the Group II conditions. Further attempts have been made to confirm the finding that various tissues of the tuberculin-sensitive animal are affected *in vitro* by tuberculin or by the 'purified protein derivatives' (PPD) of the tubercle bacillus. Skin has been used especially, since this tissue can be cultured in a state of active metabolism and growth. But it now seems that epidermal cells at least are quite unaffected in this kind of experiment, continuing to grow (Cruickshank, 1951) and use oxygen at normal rates (Cruickshank, 1954) in the presence of PPD concentrations which would cause intense reactions if introduced into the skin of the intact animal (see also Everett *et al.*, 1952). One presumes therefore that the typical tuberculin reaction involves primarily those wandering cells concerned also in the passive transfer of the reaction, and that from them are liberated potent substances affecting blood vessels and so indirectly, in extreme instances, leading to death of local areas of epidermis. Certainly, the cellular infiltration is an essential part of the reaction, failing to appear fully when cortisone or ACTH suppression is employed. The cellular infiltration in the tuberculin reaction is so intense and persistent, involving cells of a kind such that the late phases of the reaction have been likened to the

some ways to a new

It has long been

Group I and Group II types of hypersensitivity may depend on the route of administration and the manner of absorption of the various antigens involved. A condition resembling Group I hypersensitivity—the anaphylactic state—can be induced by injection of simple protein antigens Even substances commonly used to evoke Group II reactions, like tuberculin itself, lead on repeated injection into normal animals to Group I anaphy-

lactic-type hypersensitivity. To induce Group II type hypersensitivity to tuberculin, either living tubercle bacilli or suspensions of dead bacilli mixed with an oily adjuvant must be used. Similarly, some chemicals such as picryl chloride or 'tetryl' are liable after repeated application to human skin to induce contact dermatitis—a typical Group II sensitivity, but when coupled chemically with proteins and injected as these conjugates, the same chemicals give rise to specific anaphylactic hypersensitivity. As most of these chemicals couple with animal proteins at body temperature, injection of the chemical alone into suitable sites may lead to mixed hypersensitivity with characteristics both of Groups I and II. It has been suggested that surface applications produce contact dermatitis especially, as in that way the chemical can react predominantly with the –SH or –S–S– groups which are abundant in the epidermis (Eisen and Belman, 1953). But surface application with most chemicals is a slow and uncertain way of inducing hypersensitivity in normal animals. Chase (1954) has shown that preliminary injection of picryl-chloride-erythrocyte-stroma together with dead tubercle bacilli, oil and emulsifying agent, followed after a due interval by surface applications, leads in the guinea-pig to a very intense degree of hypersensitivity closely resembling human contact dermatitis. Although no obvious relation exists between this manner of sensitization and the genesis of the natural human disorder, these highly sensitive animals are likely to be of great use in future experiments designed to work out more fully the mechanisms of induction and transfer of contact hypersensitivity. Cruickshank and Howard-Swaffield (1953) in the course of investigating an outbreak of dermatitis in the laboratory of a synthetic-fibre spinning factory, have encountered a chemical (methyl octyl benzene sulphonate) which induces sensitivity in the guinea-pig after a single application to intact shaven skin.

New Methods applicable to the study of Antigens and Antibodies

In asthma and hay-fever, specific sensitizing antibodies circulate in the blood. The exact relationship between these plasma

proteins and those with the properties of protective antibodies which are to be found in human γ-globulin needs to be discovered; this information, one may feel, would help to explain how these hypersensitivity diseases arise. The antigens which evoke the symptoms (pollens, animal dandruff, etc.) are also probably proteins—at any rate they have in many instances been shown to contain molecules in the molecular weight-range 10,000–50,000 and capable of acting as precipitin-producing antigens when injected into animals The purification and characterization of these substances is also of practical and theoretical importance, and likely to prove useful in improving diagnostic methods, such as skin-testing, or specific treatment aimed at 'desensitization'. The new methods which are being applied to the study both of sensitizing antibodies and specific antigens have been developed both by workers in the general field of macromolecule studies and by immunologists.

(*a*) *Separation by electrophoresis.* It is now more than twenty-five years since Tiselius developed an apparatus for the separation in solution of charged macromolecules such as proteins. The importance of this work was quickly recognized, and the results of this form of analysis of serum globulins are firmly embedded in current terminology—γ-globulins, for example, meaning the group which migrates most slowly in an electrical field in the range of pH commonly used (e.g. pH 7·5–8 5). But the application of the method to the analysis and separation of proteins was limited by the expense of the apparatus and the length of time required for preliminary preparation of solutions to be tested, until von Turba and Enenkel (1950) described the satisfactory separation of proteins on filter paper. The value of this method in medicine has been so obvious that it is now widely used in clinical pathology and biochemistry Paper electrophoresis is applied without preliminary dialysis or other prolonged preparation. The apparatus is inexpensive, results are obtained within twelve hours, and with suitable precautions, can give accurate quantitative information (e g. Hardwicke, 1954). The paper strip with the separated protein fractions can be longitudinally bisected, part being stained for protein and part for associated substances such as carbohydrate, lipid or

metals; this may give useful accessory indications to the nature of a particular protein fraction. Similarly a part of the paper can be stained for protein bands in the usual way, and a part used for elution of small amounts of the separated fractions. Enough protein can be obtained, for example, after electrophoresis of a crude specific antigen preparation for use in skin tests on an asthmatic patient, and from any protein mixture eluted fractions can be tested by precipitin reactions. For preparative fractionation, however, when larger quantities are required, it is necessary to carry out electrophoresis on columns, such as starch, which can be cut into segments.

(*b*) *Precipitin reactions after diffusion through gels.* The use of the antigen-antibody reaction has long been known to be the most specific way of distinguishing between different molecules which are antigenic. Of the various kinds of antigen-antibody reaction that have been studied, the most direct and easily applied is the *precipitin reaction*. The quantitative finding, that these reactions occur best in any system at a certain ratio of antigen to antibody, was described by Dean and Webb in 1926. This property is the basis of an ingenious method introduced in 1947 when Oudin (1952) showed that antigen and antibody would react to form specific bands of precipitate after diffusion toward one another in an agar gel. In the original method antibody was incorporated into gelatin which was poured into the lower part of a test-tube and allowed to solidify A second layer of gelatin-containing antigen was then poured on top of this layer and after some hours, during which diffusion took place from one layer of gelatin into the other, a band of precipitate could be observed If the antigen consisted of several components to which antibodies were present in the lower layer, a number of bands appeared from which the minimal number of antigens present could be deduced. Sometimes, of course, by coincidence the diffusion rates and optimal proportions would be such that what appeared to be a single band in reality consisted of two different forms of specific precipitate occurring at the same position This difficulty could usually be overcome by using, in parallel tests, antisera prepared from several different individual animals when it would be extremely

unlikely that in every case the band fronts would superimpose. Since this technique was first introduced a number of improvements have been made. In the first place an additional layer of gelatin containing neither antigen nor antibody was interspersed between the two layers mentioned above (Oakley and Fulthorpe, 1953). This allowed more space through which diffusion occurred, lead to the reactions being evident as finer lines and increased the possibility of multiple precipitates being separately discernible. A great increase in the elasticity of the method resulted when it was carried out by horizontal diffusion through a gel contained in an ordinary bacteriological petri dish. Not only is this system very convenient in that antigen and antibody can be introduced in fluid form in cavities cut out of the agar (as is used in assaying sensitivity of organisms to antibiotics in routine bacteriology); it also allows direct comparisons to be made between the precipitin reactions resulting from diffusion of two or more
a single antibody space.
work of Ouchterlony (1
toxins produced by various strains of growing bacteria as demonstrated by their interactions with an antitoxin preparation contained in a central gutter of the agar plate on which streak cultures were growing. The technique has been applied to the investigation of serum and other protein mixtures particularly in Paris by Kaminski and Ouchterlony (1951) and by Grabar (1954a), and in Birmingham by Gell (1955). The system is so versatile that several examples of its use will be given in the following discussion

(*c*) *Combined electrophoresis and precipitin diffusion.* Electrophoretic separation of proteins can be effected in agar, for example along the centre of a thin rectangular sheet which has been poured onto a glass or 'Perspex' support. After stopping the migration by interrupting the electric current, a lateral gutter is cut adjacent to and parallel with the centre line and then filled with appropriate precipitin-containing antiserum. In the course of the succeeding twenty-four hours, precipitin- and antigen-diffusions ensue and a series of crescentic precipitates appears round the site to which electrophoretic migration of various

method has been applied mainly to the separation of components in normal serum, in which albumin, α_1-globulins, α_2-globulins, β-globulins and one complex γ-globulin fraction can be demonstrated. But in principle, analysis of the antigens in natural protein mixtures causing hypersensitivity reactions can also be minutely examined in the same way.

(*d*) *Immunological analysis using 'coated' erythrocytes.* In a search for antibodies circulating in the plasma of tuberculous patients, Boyden (1951) introduced a new serological reaction of great sensitivity. Sheep red cells were treated with tannic acid; their surfaces were then adsorbent for protein solutions (e.g. tuberculin) to which they might be exposed. Red cells so coated reacted specifically to appropriate antisera by agglutination even in high dilution, and with antisera containing no detectable precipitins. With suitable controls and, when necessary, preliminary removal of non-specific agglutinating factors, this kind of method seems currently to be the most appropriate for trying to detect non-precipitating antibodies present in sera from certain types of hypersensitivity. Examples of the use of this antigen-coated red-cell technique (e.g. Orlans, Rubinstein and Marrack, 1953) will be discussed below. Even more specific linking of antigens to red cells has been developed by Coombs, Howard and Mynors (1953); the antigen is first combined via tetrazotized benzidine with a modified Forssman antigen which will still couple strongly to the surface of sheep red cells. The antigen still retains immunological specificity so that once again, agglutination provides a delicate indicator of antibodies directed toward these 'red cell linked antigens'. The results obtained so far (Coombs and Fiset, 1954) are promising.

Characterization and Nature of Antigens Concerned in some Hypersensitivity Reactions

The antigens which induce asthma or hay-fever in hypersensitive individuals are mostly of biological origin—plant pollens, animal emanations, mould spores, for example. They are

therefore complex, and liable to be admixed with substances playing lit[illegible] reactions. [illegible] able at pr[illegible] purposes. Standardization of potency, for instance, can only be approximate, and for treatment involving injections, it seems undesirable to introduce crude contaminants together with the active principle. Complete purification and characterization has not yet been achieved for any individual antigen. The stages of such work may be summarized as follows:

(*a*) *Chemical purification and analysis* of components soluble in physiological saline and liable to be antigenic (proteins, polysaccharides).

(*b*) *Tests in animals* to determine antigenicity of these components, and production of antisera suitable for testing purity.

(*c*) *Quantitative skin tests* on numbers of patients to determine which of the components are concerned with human disease.

(*d*) *Therapeutic trials* of components active under (*c*) (and perhaps purified after relating the findings under (*a*), (*b*) and (*c*)) to determine ability specifically to 'desensitize' hypersensitive patients.

(*e*) *Provision of a stable standard* will be achieved on the basis of the previous findings and is a necessary condition for reduplication of fully satisfactory materials for diagnosis and treatment. A freeze-dried preparation is likely to be most suitable.

These stages, though logically separable, need to develop in parallel. The integration of skilled chemical investigators and immunologists, animal experimentalists and clinical observers capable of accurate measurement is a feature of the scientific basis of medicine in this, as in other fields. For brevity, various assumptions have been made in the above sections which will be discussed at length. Solubility in physiological saline has been regarded as an attribute of the antigens responsible for respiratory hypersensitivity, as they must dissolve in nasal or bronchial secretion before exciting symptoms. Skin tests have been taken as the method of testing patients as being theoretically justifiable and satisfactory in practice. The stability of these antigens in the dry state seems probable since they are usually

dust-borne before producing effects; but there is a possibility that contaminants may, unless checked, lead to degradation in solution, especially with biological materials rich in enzymes (e.g. pollens). As illustrations of the success so far achieved, consideration will be given to three specific examples of antigens that have been intensively studied—grass pollens, house dust, and horse dandruff.

Grass Pollens

These have attracted much attention for they are responsible for more symptoms than any other group of antigens. The pioneer work of Blackley carried out on himself showed conclusively the specific effect of grass pollens in reproducing symptoms of asthma and hay-fever, the value and specificity of skin tests, and the cross-reactions obtainable with iris pollen but not with dicotyledonous pollens. Early in this century, Noon and Freeman standardized extraction procedures, and later nitrogen- or protein- (precipitable-nitrogen) estimation was proposed for standardization purposes. After the First World War, much attention was paid to pollen extracts by allergists in the U.S.A. where not only grass pollens but also ragweed pollen is notorious for producing human symptoms. In 1937, Harley, working in London with grass pollens, found that the active principle did not dialyse through cellophane membranes and consisted essentially of protein together with some polysaccharide. Various procedures which tend to destroy protein were shown to reduce but not to abolish the capacity of pollen extracts to elicit skin reactions in patients. Recently the multiplicity of antigens in pollens has been emphasized. For example, Becker and Munoz (1949) have prepared antisera by injection of pollens into rabbits. On the simple Oudin precipitation technique five bands of precipitate were found showing that at least five antigens were present in the ragweed pollen which they used These rabbit antisera were furthermore shown to contain precipitins for the constituent actually causing human hypersensitivity as the supernatants after the sera had reacted with the pollen extracts gave considerably reduced reactions in the patients who were tested. This kind of precaution is

necessary since obviously a particular dust or pollen may contain proteins antigenic for a rabbit after injection but not in fact concerned with any human symptoms. Gosselin, Mynors and Coombs (1953) have used the coated red cell agglutination technique for showing the specificity of pollen extracts. Antisera prepared in rabbits could be shown specifically to agglutinate these red cells though cross reactions were such that within a group of six grass pollens of different species found in English meadows no distinction could be made by agglutination. If, however, the antisera were submitted to adsorption tests with separate preparations of pollens, quantitative specific agglutination could be shown for each pollen separately. In these tests no attempt was made to demonstrate that each protein component of the grass pollens was in fact responsible for human hypersensitivity reactions, but in view of the presence of these species-specific proteins the possibility remains that a patient might be specially sensitive, say, to Timothy grass and little if at all to, say, rye grass.

More lately various workers have emphasized that some degree of skin reaction can be obtained from low molecular weight derivatives obtained from pollen extracts. Frankel and others (1955) for example, have studied peptides and low molecular weight polysaccharides which can be separated from the main pollen extract by dialysis and then purified by chromatography on paper using a solvent such as methyl cellosolve. In this way a number of peptides were found to give positive reactions of roughly equal sizes though in every case apparently much less than the non-dialysable main pollen component. Much more work needs to be done before the significance of these findings is clear On general biological grounds it might be thought that pollen proteins would be difficult to purify, since from their function the pollen grain must be capable of spontaneous breakdown in order to form the tube concerned in seed fertilization. If pollens are indeed liable to 'autodigestion', degradation to peptide and polysaccharide fragments from the main antigen may readily occur. Other workers have emphasized the need for using a fairly intact pollen protein for desensitization purposes. For example, Frankland and Augustin

(1954) carried out a very carefully controlled test of the prophylaxis of summer hay-fever. Four separate solutions were used in treatment: (*a*) standard whole pollen extract ('pollacine'), (*b*) pollen protein carefully purified by dialysis, (*c*) an ultrafiltrate from the 'pollacine' containing smaller polysaccharides and some nitrogen, and (*d*) a control solution of the suspending fluid, phenol-saline. The results from one season's treatment with these four extracts were clear-cut. Good or excellent desensitization results were obtained in 16 out of 17, and 13 out of 14 instances of treatments (*a*) and (*b*) respectively. With the ultrafiltrate and with the control solution only about one third of the patients said they had been benefited. Augustin (1953) is clear that the active principle in grass pollen is a protein, containing only about 2 per cent of carbohydrate, has a molecular weight of about 14,000, and is antigenic in guinea-pigs and rabbits from which good precipitating antisera can be obtained. The chemical and immunological properties of Timothy grass pollen extracts has been well reviewed by Augustin-Friedmann (1952).

House Dust

[illegible] Maunsell [illegible] ible for [illegible] is antigen. The technique used consisted of adsorption onto benzoic-acid crystals, precipitation with 80 per cent acetone, followed by dialysis and reprecipitation with acetone. No loss of activity was found on treating the purified dust antigen with alkaline trypsin or various other enzymes. Boiling with 0.5 NHCl for half an hour resulted in breakdown of nearly all the carbohydrate, but only minimal liberation of amino-nitrogen, biological activity being apparently unimpaired. Further hydrolysis however leads to liberation of aminoacids and loss of activity. In the active, partly degraded material, eleven aminoacids (nearly all simple mono-aminoacids) and the sugar galactose were found. This 'chemically modified antigen' contained only about 10 per cent carbohydrate. Electrophoretic analysis separated three fractions, all of which elicited positive skin reactions.

The purified house dust antigen was found to be satisfactory for diagnostic tests *(intradermal, 0·04 ml.) in dilutions of* 10^{-5} to 10^{-8}. Only 4 per cent of normal individuals reacted to the strongest solution used for diagnosis. Satisfactory 'desensitization' was achieved with this material. As sensitivity to house dust appears to be common, at any rate in London, this work is of great clinical interest, and immunological analysis with animal antisera as a guide to further purification would be well worth carrying out.

HORSE DANDRUFF

Some preliminary purification of this antigen was achieved by Squire (1950) using dialysis and salt precipitation. The active material was shown to be a protein, destructible by pepsin in acid solution, and having the salt solubility of an albumin. Dandruff protein was a good antigen in rabbits which yielded potent precipitating antisera. With these antisera, and in skin tests on patients, cross reactions with purified horse serum albumin were obtained. Stanworth (1953) confirmed and considerably extended these findings Electrophoresis in the Tiselius apparatus and on paper showed the presence of at least three protein fractions in crude extracts, some being rich in carbohydrate. Potency in skin tests appeared to be concentrated in one electrophoretic fraction, and partially-purified pigment-free, freeze-dried preparations were obtained, the approximate molecular weight of one of these being 35,000. Using the Oudin technique, three bands of precipitate were demonstrable when crude preparations were tested with rabbit antisera, and the progress of purification could be assessed in this way. A crystalline protein preparation was even obtained from horse dandruff, but this did not appear to contain or to retain the full degree of activity of the crude material on skin testing. This work is therefore proceeding

To summarize, notable advances have been made, especially in the last decade, toward the goal of a pure antigen concerned with human hypersensitivity disease, but much ground remains to be covered.

(1954) carried out a very carefully controlled test of the prophylaxis of summer hay-fever. Four separate solutions were used in treatment: (*a*) standard whole pollen extract ('pollacine'), (*b*) pollen protein carefully purified by dialysis, (*c*) an ultrafiltrate from the 'pollacine' containing smaller polysaccharides and some nitrogen, and (*d*) a control solution of the suspending fluid, phenol-saline. The results from one season's treatment with these four extracts were clear-cut. Good or excellent desensitization results were obtained in 16 out of 17, and 13 out of 14 instances of treatments (*a*) and (*b*) respectively. With the ultrafiltrate and with the control solution only about one third of the patients said they had been benefited. Augustin (1953) is clear that the active principle in grass pollen is a protein, containing only about 2 per cent of carbohydrate, has a molecular weight of about 14,000, and is antigenic in guinea-pigs and rabbits from which good precipitating antisera can be obtained. The chemical and immunological properties of Timothy grass pollen extracts has been well reviewed by Augustin-Friedmann (1952).

House Dust

Very great progress has been made by Rimington and Maunsell (1950) in purifying the principle in house dust responsible for positive skin tests in patients with hypersensitivity to this antigen. The technique used consisted of adsorption onto benzoic-acid crystals, precipitation with 80 per cent acetone, followed by dialysis and reprecipit [illegible]
was found on treating th [illegible]
trypsin or various other [illegible]
half an hour resulted in [illegible]
drate, but only minimal liberation of amino-nitrogen, biological activity being apparently unimpaired Further hydrolysis however leads to liberation of aminoacids and loss of activity. In the active, partly degraded material, eleven aminoacids (nearly all simple mono-aminoacids) and the sugar galactose were found. This 'chemically modified antigen' contained only about 10 per cent carbohydrate. Electrophoretic analysis separated three fractions, all of which elicited positive skin reactions.

mixed with appropriate antigens in the presence of such cells, a more convenient alternative *in vitro* test would be available. An example of such release in an experimental situation has been provided by Humphrey and Jaques (1953) who contrasted the reactions of antigen and purified precipitins with analogous reactions of antigen and precipitin-containing plasma. In the latter instance, quantities of serotonin-like substance and histamine were released probably as the result of platelet disintegration. Histamine-release from hypersensitive skin *in vivo* on exposure to antigen has been demonstrated by Katz (1942) but attempts by other authors to reproduce this effect *in vitro* have not shown such large differences between sensitized and normal skins, so that these results do not seem to suggest a reliable test. Schild and others (1951) however found that histamine was released *in vitro* from the bronchial preparations mentioned above when exposed to the specific antigens.

(*c*) *Using coated red cells*, Orlans, Rubinstein and Marrack (1953) carried out a series of tests on the sera of patients suffering from hay-fever. Although the pollen-extract-coated red cell suspensions were satisfactory, giving no agglutinations with twenty normal sera diluted 1 in 6, only six out of twenty-two untreated hay-fever patients' sera gave positive results at this dilution. Following a course of desensitization 100 per cent of the sera caused agglutination, in many instances at high dilutions. The authors therefore concluded that this test is not satisfactory; the antibodies detected following 'desensitization' were presumably of the protective or 'blocking' type and not of the type concerned with hypersensitivity. Coombs, Howard and Mynors (1953) used the red cell linked antigen test and obtained some specific positive results with the sera of patients with hypersensitivity to horse dandruff; parallel tests with coated tanned red-cell suspensions gave negative results. In further tests Coombs and Fiset (1954) studied methods of detecting complete and incomplete antibodies to egg albumin. This report shows that the method can be so sensitive that the sera of persons with no symptoms from eating eggs may nevertheless react so as to indicate the presence of incomplete antibodies This technique therefore is to be regarded as even more sensitive

Nature of Circulating Antibodies concerned in some Forms of Hypersensitivity

The Prausnitz-Kustner reaction provides a certain basis for proving the presence of circulating antibodies, apparently in steady concentrations for periods of years, in such conditions as asthma and hay-fever. But the practical use of such a test, involving the injection of serum or plasma into the skin of a normal volunteer is limited, especially now there is knowledge of the theoretical risk, albeit small, of transmitting serum jaundice. Recent work on the nature of these circulating antibodies has therefore been directed towards (*a*) discovering, if possible, an *in vitro* test for their presence, and (*b*) locating more accurately their properties within the globulin fractions of serum and defining their relations with other types of circulating antibody in hypersensitivity states and in contrasted infective diseases.

In Vitro Tests

(*a*) *Using isolated bronchi* removed from a young patient with bronchiectasis and with bronchial asthma, Schild and others (1951) were able to demonstrate smooth muscle contraction on exposure to specific antigens known to give positive skin reactions in the same patient. This finding is analogous to the classical experiments with guinea-pigs rendered anaphylactically sensitive; isolated gut or uterine strips from these animals contract when exposed to the specific antigen. The success of Schild and his collaborators leads to the question whether the bronchi removed from non-sensitive pateints (e.g. with lung carcinoma) could be specifically sensitized by soaking in the serum or plasma from hypersensitive patients. If so, not only could the diagnostic skin tests currently used be replaced by a laboratory procedure, a suitable experimental technique would be available for testing fractionated hypersensitivity sera and for making other kinds of experimental advances.

(*b*) *Release of active bases* such as histamine and serotonin (5-hydroxytryptamine) has long been sought when antigen-antibody reactions take place in the presence of other cells such as leucocytes, platelets or skin slices. If this could be unequivocally demonstrated, using hypersensitivity antibodies

total) was distinguished on the basis of individual and family history of symptoms. As was to be expected, therefore, the correlation between history and type of reaction to immunization was significant statistically, rather than a perfect correspondence.

Kuhns (1954) has extended these findings by electrophoretic separation on starch columns of the sera from both types of individual after immunization with diphtheria toxoid. The columns were transected into some thirty segments and the protein concentration in each was estimated. From the graphical plots in Kuhn's paper, it is easy to see the separation into what are presumably, α_1-, α_2-, β-, and γ-globulins, the last fraction being spread over more segments than any other fraction. As expected, nearly all of the antitoxin (assayed as animal protection units) was found in this γ-globulin fraction In some instances, however, it was mainly to be found in the trailing edge of this fraction, sometimes called the γ_2-globulin. In these instances, the subjects were not hypersensitive and the antitoxin had ordinary precipitating qualities. In others, the antitoxin was concentrated in the leading edge of the γ-globulin fraction (γ_1-globulin); this kind of antitoxin gave but little precipitate, and was usually obtained from subjects who developed skin hypersensitivity. Again, individuals with both forms of antitoxin were found, but in them, the γ_2-globulin antitoxin was of the precipitating form, while the γ_1-globulin was comparatively poor in precipitating qualities.

One may now attempt to correlate these findings with our current knowledge of the composition, electrophoretic mobility and immunological properties of γ-globulin in normal and in other forms of hypersensitivity In a recent review, Grabar (1954b) has pointed out that γ-globulin in man and animals is composed of more than one entity, in the ultracentrifuge, especially, at least three major components can be separated; they can broadly be described as relatively small, medium and large macromolecules. On electrophoresis, however, no clear-cut separation occurs. On electrophoretic paper strips, for instance, a broad continuous area is seen running up close to the comparatively well-defined β-globulin band. When electrophoresis is combined with precipitin diffusion—a technique

than procedures using coated red-cells, but it appears that a 'happy medium' or altered procedure would need to be worked out if accurate differentiation and diagnosis of hypersensitive states were to be made in this way. Britton and Coombs (1955) have used both 'coated tanned red-cell method' and the 'red-cell linked antigen test' to detect specific heat-stable antibodies presumably of the blocking antibody type in patients who had been 'densensitized' by treatment. In any case, the handling of these special red-cell suspensions remains at present an expert procedure, unsuitable for widespread application. The general problem of 'non-precipitating antibodies' including their detection and significance has been reviewed by Marrack (1951). A negative definition ('non-precipitating') is always liable to be confounded by further discoveries, and there remains the possibility that under the right conditions, the serum or plasma of a hypersensitive patient could give rise to a specific precipitate. Marrack and Grant (1953) by using the increased scattering of light or passage through antigen-precipitin mixtures under conditions of low salt concentration in which true precipitation does not occur, were able to show that interactions nevertheless took place.

Sensitizing Antibodies after Immunization

In 1952, Kuhns and Pappenheimer reported their initial immunochemical studies of the kinds of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. The allergic individuals tended to develop hypersensitivity as demonstrated by wheal-type skin reactions when tested by intradermal toxoid. The sera of these same individuals often failed to precipitate on admixture of toxoid, in spite of good titres of antitoxin as shown by toxin-neutralization tests in animal skin. Other individuals—the 'non-allergic' group—developed antitoxin with normal precipitating qualities, and showed no signs of hypersensitivity when skin-tested These findings are of such importance that critical appraisal may be attempted The two groups of subjects were not completely divisible—some individuals showing some of the attributes of both groups. The individuals were all healthy students, and the allergic group (which formed an appreciable proportion of the

absent (hypo-, a-gammaglobulinaemia) are therefore of special interest. The details of one such patient in whose serum not even traces of γ-globulin could be detected (e.g. by precipitin diffusion through gels) have been published by Grant and Wallace (1954). As usual, recurrent infections, especially with pyogenic cocci, were noted, though prolonged remissions can occur. The infections often involve skin and the lungs, and are amenable to chemotherapy though often relapse when treatment is withdrawn. Leucocytosis in response to infection is usual, and abscess formation may occur. Tests for antibodies in the serum are usually negative (Widal, smallpox-neutralization tests, etc.) and test doses of diphtheria toxin may damage the skin (Schick test) even after attempted immunization with toxoid. Of considerable interest for the study of different types of hypersensitivity is the finding that tuberculin-test skin reactions may still be strongly positive even when complete a-gammaglobulinaemia is present. Apparently, hypogammaglobulinaemia at least may be an acquired condition, and clinical observation may eventually indicate whether a patient with asthma or hay-fever would lose this form of hypersensitivity, as one would expect, if hypogammaglobulinaemia were to develop At all events, the importance of γ-globulin in conferring protection against many forms of infection is well attested, both by the findings in states of deficiency and by therapeutic success by conferring passive immunity in these and other patients by administration of preparations rich in γ-globulin as a form of treatment.

Some idea of the mechanism by which the precipitating antibodies in γ-globulin act within the body is provided by the experiments of Talmage and others (1951) and by those of Laws and Wright (1952). Talmage and others used ^{131}I-labelled bovine γ-globulin and studied its rate of disappearance from the blood stream of rabbits. Some of these animals had been 'sensitized' by recent injections of bovine γ-globulin; others were normal controls. The rate of removal of the radioactive foreign protein was slow in the controls—4 per cent of the foreign protein still circulating after 4-5 days, the rate was much more rapid in the 'sensitized' group—less than 1 per cent

described above, the γ-globulin precipitate, though continuous, extends straight forwards across the symmetrical crescents of precipitate corresponding to β_1-, β_2-globulins, even approaching the region of α_2-globulins. These findings may explain the discrepancies reported by several authors (e.g. Menzel *et al.*, 1952, Vaughan and others, 1952), in trying to locate the sensitizing antibody responsible for Prausnitz-Kustner passive transfer reactions obtained with fractions derived from patients with asthma or hay-fever. This antibody, variously regarded as γ-, β- or even α_2-globulin, is probably also in fact a form of γ_1-globulin tending to migrate more rapidly on electrophoresis than the remainder of the γ-globulin fraction and perhaps in consequence, differing somewhat in solubility characteristics.

The γ-globulin content of the sera of patients with various forms of hypersensitivity merits further examination. By paper electrophoresis, Hardwicke (personal communication) has found the concentration raised above normal in some children with asthma, but careful repeated studies have not yet been done to avoid confusion due to intercurrent infection and other complicating factors. The same investigator has studied a severe example of serum sickness following the administration of 20 ml. of horse serum anti-snake venom to a previously sensitized individual for the treatment of symptoms caused by an adder-bite. Acute rises occurred in the concentration of all plasma protein fractions except albumin (which fell somewhat). The γ-globulin continued to rise for fourteen days following the administration of horse serum reaching a concentration of more than 2 g./100 ml. Four months previously the γ-globulin concentration was normal (1·0 g./100 ml.) but fifty-two days after dosage with serum, the level was still 1·5 g./100 ml., all other fractions including the fibrinogen having reverted to normal within twenty-eight days. There is little doubt that some form of γ-globulin is intimately concerned in many kinds of hypersensitivity state.

Biological Significance of Different Types of Antibody

Most forms of antibody in man appear to be carried in the γ-globulin. Conditions in which this fraction is deficient or

AUGUSTIN-FRIEDMANN, R. (1952). *Quart. Rev. Allerg* **6**, 290
AUGUSTIN, R. (1953). *Biochem J.* **54**, xP.
BECKER, E. L. and MUÑOZ, J. (1949). *Proc Soc. exp. Biol , N.Y.* **72**, 287.
BOYDEN, S. V. (1951). *J. exp. Med* **93**, 107.

COOMBS, R R. A. and FISET, M L. (1954). *Brit. J. exp Path* **35**, 472.
COOMBS, R R. A , HOWARD, A. N. and MYNORS, L. S. (1953). *Brit. J. exp Path* **34**, 525.
CRUICKSHANK, C. N. D (1951) *Nature, Lond.* **168**, 206.
CRUICKSHANK, C N D. (1954). *Brit J exp. Path.* **35**, 609
CRUICKSHANK, C N. D and HOWARD-SWAFFIELD, H. (1953). *Brit. J. industr. Med.* **10**, 121.
DEAN, H. R. and WEBB, R. A. (1926). *J. Path. Bact.* **29**, 473.
EISEN, H N. and BELMAN, S. (1953). *J. exp Med.* **98**, 533.
EVERETT, E. T., LIVINGOOD, C S., POMERAT, C M. and HU, T. (1952). *J. invest. Dermat* **18**, 193.
FRANKEL, S , JOHNSON, M. C , SCHICK, A W , BUKANTZ, S. C and ALEXANDER, H. L. (1955). *J. Allergy*, **26**, 33.
FRANKLAND, A. W. and AUGUSTIN, R (1954) *Lancet*, **1**, 1055
GELL, P. G H (1953). *Biochem Soc. Symp.* **10**, 63.
GELL, P. G H (1955) *Biochem. J* **59**, viiiP
GELL, P. G H. (1955) *J. clin. Path.* **8**, 269
GELL, P. G. H. and HINDE, ISOBEL T (1951) *Brit J exp Path.* **32**, 516
GOSSELIN, M. L , MYNORS, L S. and COOMBS, R. R A (1953) *Acta allerg., Kbh.* **6**, 96

758.

and
375
LAWS, J. O and WRIGHT, G P (1952). *Brit. J exp Path* **33**, 343
MARRACK, J R (1951). *Int. Arch Allerg , N Y.* **2**, 264
MARRACK, J R and GRANT, R A (1953) *Brit J exp. Path* **34**, 263
MENZEL, A E O , KESSLER, W R , COOKE, R A and MYERS, P (1952) *J Allergy*, **23**, 483
OAKLEY, C L and FULTHORPE, A J (1953). *J. Path Bact.* **65**, 49.

remaining after 24–36 hours. Laws and Wright injected rabbits with radioactive bovine albumin and confirmed the difference between its rate of disappearance from the blood in normal and immune animals. In the latter, intravascular precipitation presumably occurred and radioactive foreign protein which continued to circulate in the normal animals, was concentrated in the spleen, liver and other organs rich in fixed reticulo-endothelial cells. Furthermore, radioactive iodine was excreted into the urine by the immune animals much more rapidly than by the normals. Not only was the foreign protein removed from the blood more rapidly; once taken up by reticulo-endothelial cells, apparently, it was digested into fragments small enough to be excreted or metabolized. This kind of work provides a satisfactory experimental basis for assessing the functional significance of precipitating antibodies. It is in accord with classical experiments on phagocytosis of bacteria by leucocytes in the presence of specific antisera. For non-precipitating antibodies and for the γ_1-globulin sensitizing antibodies sometimes found by Kuhns and Pappenheimer (1952) in normals immunized with diphtheria toxoid, no such function is apparent. Some protective function is evident from the efficacy of this antibody also as an antitoxin. Is this non-precipitating antibody a poor form (an 'incomplete' antibody), or does it possess some specialized function? One may envisage, for example, the large precipitating γ-globulin molecules are by their nature relatively confined to the blood-stream where they remain ready to assist in the removal of foreign substances, whereas a function of possibly smaller γ-globulin antibodies may be to diffuse more readily into the interstitial fluid and so to act (perhaps in conjunction with wandering cells) at a different site The further analysis of this variety of antibody may show whether some types of hypersensitivity are indeed due to an exaggerated normal response and if so, perhaps lead to suggestions for their control.

REFERENCES

Ackroyd, J F (1949). *Clin. Sci* 7, 249.
Ackroyd, J. F. (1954). *Clin Sci.* 13, 409

IX

Protein Malnutrition

B. S. PLATT

SINCE the second World War there has been increased interest in proteins and their metabolism and it is safe to prophesy that a still greater interest in the role of these substances in biology and medicine will develop Proteins have long been recognized to be the most important of all known substances in living tissues—animal and vegetable; indeed, the name given to them in 1839 by the Dutch chemist Mulder is derived from the Greek word 'proteios' meaning 'primary' or 'first'. Mulder wrote at that time, 'Without it (protein) no life appears possible on our planet. Through its means the chief phenomena of life are produced.'

Although the study of proteins has been continuous for over a century and much progress has been made, it was somewhat eclipsed between the two World Wars by research on vitamins. Already in retrospect it is becoming clear that just as vitamin research derived an impetus from the first World War, so protein research has been stimulated by developments during and since the second World War; three prominent ones are:

(1) the attention devoted by national and international agencies to the nature and prevalence of some forms of protein malnutrition contributing to this development are the observations on, and sometimes the personal experiences of, prisoners of war; investigations of the effects of post-war food shortages on human health, and the urgency of the need for increasing food supplies to meet the nutritional needs of the world population, which is expanding at an unprecedentedly high rate;

ORLANS, E. S., RUBINSTEIN, L. J. and MARRACK, J. R. (1953). *Acta allerg., Kbh.* 6, 33.

[illegible] , ed. Corcoran, A. C.

RIMINGTON, C. and MAUNSELL, K. (1950). *Int. Arch. Allerg. N.Y.* 1, 115

SCHILD, H. O., HAWKINS, D. F., MONGAR, J. L. and HERXHEIMER, H. (1951) *Lancet*, ii, 376

SQUIRE, J. R. (1950). *Clin Sci.* 9, 127.

SQUIRE, J. R. (1952). *Brit. med. J.* i, 1.

STANWORTH, D. R. (1953), Ph.D. thesis, Univ., Birmingham.

TALMAGE, D. W., DIXON, F. J, BUKANTZ, S. C. and DAMMIN, G. J. (1951) *J. Immunol.* 67, 250.

TURBA, H VON and ENENKEL, H. J. (1950). *Naturwissenschaften*, 37, 93.

VAUGHAN, J. H., FAVOUR, C B. and JAFFEE, I. H. (1952). *J. Allergy*, 23, 489.

to include disturbances or abnormalities of nutrition from causes other than those due to imperfections in the diet—such malnutrition will be called 'secondary' or 'conditioned' malnutrition

Clearly one way of detecting dietary protein malnutrition is to determine the differences between protein intakes and requirements. Unfortunately dietary requirements for protein are not known with precision. We know that there are two main categories of needs, (i) for maintenance in the normal adult, and (ii) for growth in childhood, in the mother for foetal growth and for the preparation for and continuation of milk secretion during lactation.

More data are needed on human requirements for protein;[1] these data might best be expressed in terms of amino-acids; more analytical data are needed on the amino-acid composition of the proteins of foods. Our information on the digestion and fate of proteins in the body is incomplete and we need to know more about (*a*) the suitability of protein for various purposes, (*b*) the effects on the nutritive value of proteins of procedures employed in the processing and sophistication of foods, and (*c*) the interrelationships in human nutrition between proteins, peptides and amino-acids and certain accessory food factors and minerals.

In this lecture I shall not have space to discuss the differences in the amino-acid contents and the biological values of proteins. I would however like to emphasize that some amino-acids are intimately related in metabolism to certain B-vitamins; that it has been shown that all the amino-acids for a given synthesis must be at the site of anabolism in the body in the amounts required and at the same time, and that dietary proteins differ in value for such varied purposes as the synthesis of haemoglobin, of plasma albumin and the regeneration of granulocytes. The forms of malnutrition which I shall consider are summarized in the following chart:

[1] The [illegible]

[illegible]

Organization, 1955)

(ii) the recognition of the life-saving value of blood and plasma transfusions—in the treatment of war wounds, injuries, burns and in surgical procedures, as one of the triumphs of present-day therapeutics; it is not always remembered that proteins constitute over 90 per cent of the total solids of whole blood, about 80 per cent of those of plasma and that a litre of blood contains about 200 grams of protein;

(iii) atomic research and developments which have provided a variety of isotopes, the use of which has increased the range and volume of investigations into protein metabolism.

A catalogue of the factors that have contributed to progress in the study of proteins would also include:

(i) the isolation, characterization and studies of the structure of proteins by physiochemical means, e.g. by electrophoresis, by different types of spectrometry and by electron microscopy;

(ii) discovery of new methods of identification and estimation—especially by chromatographic techniques, of the amino-acid components of proteins;

(iii) the advancement of the study of the role of proteins in immunity against disease;

(iv) the growth of knowledge of the nature and properties of enzymes, genes and viruses;

(v) the collateral development of knowledge of the chemistry of micro-organisms—especially of the effects of antimetabolites, antibiotics, and other chemotherapeutic agents;

(vi) discoveries—which are probably only the forerunners of many more—of the relationship between the anabolism and catabolism of protein and the activity of several endocrine glands.

Definitions

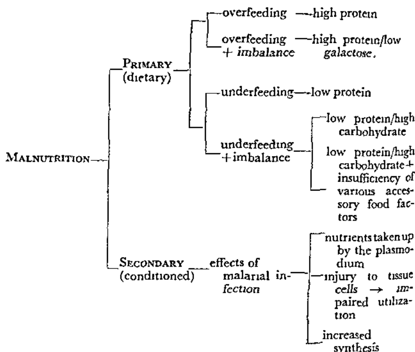
Discrepancies between the body's needs for protein and amount of protein in the food eaten give rise to 'primary' or 'dietary' malnutrition; this type of protein malnutrition may be accompanied by imbalance of the diet with respect to a wide range of other nutrients. The term malnutrition, however, will be used

When I first recalled these observations on the chemical composition of the human infant in a criticism of artificial methods of infant feeding (Platt, 1954a) I was somewhat more diffident about them than I am now, in view of recent reports (Lowe *et al.*, 1954; Creery and Neill, 1954) on the occurrence of the syndrome of hypercalcaemia in infants. There are, for calcium content of the bodies of the human infant, data analogous to those for nitrogen (Chart 2, Jeans, 1952) from which it may be seen that the calcium concentration in the body of the cows' milk fed infant is as much as 50 per cent higher than that of a naturally fed one. The only factor common to all the reported examples of hypercalcaemia in infants is that, for the most part of its life, the affected infant had been fed on cows' milk, which contains four to six times as much calcium as in human milk.

May I draw attention to an old observation (Table 1, Platt and Moncrieff, 1947) which dates back to the chemist von Bunge (1874) who showed that the times taken for a new-born animal to double its birth weight form a series which runs parallel with the values for the concentration of protein in their mothers' milk. The human infant grows exceptionally slowly—the ratio of the time it takes to attain puberty to the length of the normal span of life is uniquely high in comparison with that for all other animals. Human milk contains the least concentration of protein of all milks; cows' milk is next on the list; but the latter has a much higher total protein content and it would seem to be 'designed' for making meat more quickly than is natural in the human baby. With all due regard for the desirability of increasing rates of production of meat in animal husbandry, I doubt if such a goal is desirable in human nutrition.

Evaluation of the possible effects of cows' milk feeding of infants on health and well-being in adult life will be difficult, and obviously, from the nature of the problem, require many years of observation. In the meantime, I suggest that high protein feeding in infancy may set the infant's nitrogen metabolism in a high—an abnormally high—gear whilst, as I shall indicate later, the nitrogen metabolism of an under-fed infant may achieve a 'steady state' at an unusually low level There is evidence (Dole *et al.*, 1953) of a connection between increasing

EXAMPLES OF PRIMARY (DIETARY) AND SECONDARY (CONDITIONED) *MALNUTRITION*



OVERFEEDING WITH PROTEIN

Excessive overfeeding—or overeating—is now recognized as a form of malnutrition. Protein overfeeding may occur with cows' milk as a substitute for breast milk for feeding human infants. There is evidence (Chart 1, Jeans, 1952) that the percentage of nitrogen in the body of the cows' milk fed infant *is consistently* high[1]—at nine months of age it is approximately twice that of the breast-fed infant. I cannot find any judgement of the value of this consequence of cows' milk feeding; it has lain unquestioned for many years. We should know if the nitrogen-stuffed *baby is necessarily the prize baby—is the biggest the best?*

[illegible] content of the cows' milk

stomach, but that they might have different origins and different fates from the curd. I cannot at present relate these findings to the behaviour of milk in the stomach of the human infant. The possibility that a somewhat similar procedure may occur is supported by the observation that a blood clot layers in the human stomach in the manner I have described above for milk. It is, I admit, difficult to believe, if this phenomenon occurs in the stomach of the human infant, that it has escaped notice. I would, however, point out that the techniques for studying the behaviour of food in stomachs—by radiology and by intubation—are almost invariably applied only after the stomach has been emptied; this procedure in my experience with baby rats completely upsets the normal pattern for several hours. Also, until quite recently, infants on whom studies might have been made have been fed 'by the clock' and not 'on demand' as is usual in primitive communities and seems to be the natural way for mammals. However, whether or not a similar arrangement holds for rat and human infant, it seems worthwhile examining the possibility arising out of these observations on rats—that breast feeding of infant animals may be an intermediate stage between complete dependence on the mother before birth and complete independence after normal weaning from the breast, and that the mother may provide in the milk—and especially, if not exclusively, in the whey fraction—nutrients which the infant cannot synthesize for itself. Such substances would be 'essential' or 'indispensable' nutrients analogous to the essential amino-acids and certain accessory food factors.

The constituent of milk which passes most completely into whey is milk sugar, lactose, a disaccharide composed of one molecule of glucose and one of galactose. As a component of a cerebroside molecule, galactose is found widely distributed throughout the body in small amounts but it is especially abundant in myelin It is also found as a polymer, galactogen, in lung, as a component of glycoproteins in plasma globulins, and it is found in mucopolysaccharide present in the connective tissue, e.g. of

protein levels in the diet and the size of the appetite, which *might be so much increased as to lead to excessive overeating.* One possible consequence of an increase in the nitrogen content of the tissues may be reflected in an increased rate of muscle development. There is in fact a very wide range of time for the changes in certain forms of behaviour in the human infant; these may in part at least depend on the differences in the infants' diets though I have no information on this point. If, in turn, the more rapidly developing muscles require a correspondingly rapid myelination of the appropriate nerve supply, then there may be an increased requirement for the materials for myelin formation. Myelination of nerves in the human infant largely occurs after birth and may take five to six years to complete. Galactose is known to be an important component of one of the cerebrosides—normally the principal one of the white matter of the central nervous system. I should like now to examine the evidence for the possibility that the supply of galactose may be relatively deficient for an infant fed on cows' milk (Platt, 1955).

Cows' Milk versus Human Milk in Infant Feeding—High Protein/Low Galactose

Some new observations on the behaviour of milk in the infant rat stomach have led me to suspect a special role for galactose in infant nutrition. I have found that normally the contents of the suckling rat's stomach can be separated into layers (Platt, 1954b); these layers are comprised of the curd fractions from successive portions of feeds and they are arranged in such a way that the curd from the portion of the feed last ingested is on the outside—the clot in the centre having been in the stomach the longest time and it may be derived from a feed taken 15 or more hours before that forming the outermost layer. This arrangement of layers is the reverse of that found by von Grützner fifty years ago when he fed pellets of bread paste to rats.

The whey fraction from a portion of a feed leaves the stomach much more rapidly than the curd; about two-thirds passes out in about 40 minutes. I suspect that some of the components of whey not only behave differently from the curd portion in the

I cannot answer these questions. I can, however, offer some data which have given us a basis for research. Cerebrosides are synthesized in excessive amount in Gaucher's disease; presumably in this condition supplies of galactose are low in relation to the demands during excessive synthesis and it is found that in the spleen and sometimes in the brain of patients who had died from Gaucher's disease there is a substantial amount of glucose-containing cerebroside. Similarly, as much as 25 per cent of the carbohydrate obtained on hydrolysis of the cerebroside of the brains of rats and pigeons fed on dextrose may be glucose, whereas in the normal animal the carbohydrate fraction is principally, if not entirely, galactose (Platt, 1955).

These comments naturally lead to more questions—do these abnormal cerebrosides form in the nervous system of the artificially fed baby? Do they persist into adult life? If so, what are the consequences, if any, when stress factors are imposed in later life? Does a relatively low galactose and high protein level in the infant diet affect the biochemistry of the human body and its reaction to disease in other ways?

Protein Undernutrition

The main nutrition problem of about half the world's population is shortage of food, generally including shortage of supplies of proteins I shall attempt now to indicate the extent to which protein insufficiency may be a factor in the aetiology of a range of malnutrition syndromes.

A list of evidences of protein undernutrition in human infants and in young experimental animals would include the following (Platt, 1953a, 1954c):

(1) low birth weights and body lengths;
(2) stunting of growth and retardation of development,
(3) wasting of tissues, especially of muscle;
(4) dyspigmentation and altered texture of skin and hair;
(5) anaemia;
(6) impaired digestion and absorption of food, accompanied

I can find no evidence of the synthesis of galactose in the human body in any tissue and at any age, except in the mammary gland during lactation. This may not mean that tissues cannot make galactose but it does strengthen the case for the view that the infant might well be dependent on its food for this nutrient. There may indeed be an argument for supplying the human infant with a milk of high lactose content in view of the evidence that there is a direct relationship between the lactose content of an animal's milk and the weight of its brain and also of the length of the gestation period of the species. The correlations[1] show that man has the heaviest brain, the longest gestation period and the highest concentration of lactose in the maternal milk; cows' milk contains only about two-thirds the amount of lactose found in human milk. You will recall that of all mammalian milks, human milk has the lowest protein content—the human baby taking the longest time of all animals to double its birth weight. The ratio of protein to galactose in human milk is therefore low—certainly as compared with cows' milk; what are the effects, if any, of feeding to a baby a milk of relatively high protein/galactose ratio we can only speculate upon[2] (Platt, 1955).

The questions I have to pose here are:

(1) Is the high nitrogen concentration of the tissues of the cows' milk fed infant related to increased rate of muscle development?

(2) Does this development require an increase in the rate of myelination of the nervous system?

(3) What are the consequences of an increase in rate of myelination coupled with a relatively short supply of galactose?

[illegible] of galactose·

been published (Nagchaudhuri and Platt, 1954). This change can be produced by mild oxidation of normal hair pigment. Hair of this character can be seen on the heads of infants who have had little or no food except their mothers' milk, and similar changes can be produced in the black hair of hooded rats if they are kept on a low protein (8 per cent casein) diet. We have reported that the hair from protein deficient infants has a low cystine content and this observation has recently been confirmed. There is, however, in the deficient animal an apparent increase in -SH groups in the neighbourhood of the hair follicle —the significance of this observation we cannot explain.

The tissues from animals on low protein diets may show what I have called 'lean and hungry cells'—this appearance can be seen in a section of pancreas—Plate VI, Figure 1.[1] In Plate VI, Figure 2, the cells of another section of the pancreas from an animal which had been on a low protein diet for a long time may be said to be 'down and out'. The section of liver (Plate VII, Figure 3) from an underfed rat shows replacement of the normal cytoplasm by what appears to be glycogen.

The livers of the Gambian infants and young children on whom most of the observations recorded above have been made often showed a more or less marked fibrosis (see Plate VIII, Figure 4). None of the liver biopsy specimens showed evidence of heavy fatty infiltration; some had a mild or moderate degree of fatty change. There is a possibility that malaria may contribute to liver fibrosis[2] though a similar type of lesion has been reported

[1] [illegible] and photomicrographs Dr E. M Demaeyer kindly gave his permission to reproduce Figure 6

[2] Since this lecture was written, a report of work done in my Unit on fibrosis of the liver in West African children has been published (Walters and Waterlow, 1954) The authors put forward a theory of dual aetiology—briefly, that mal- [illegible]

These manifestations are associated with a number of biochemical and tissue changes, including:

(1) alterations in plasma protein concentration—typically reduction of albumin and generally a reduced albumin/globulin ratio;

(2) reduction in haemoglobin concentration;

[(1) and (2) may, in part only, be accounted for by haemoconcentration]

(3) low output of nitrogen in the urine with altered partition of nitrogenous components (the proportion of total nitrogen excreted as urea is reduced);

(4) reduction in plasma and liver esterase and of some other enzymes;

(5) impaired liver function, affecting conjugation of benzoic acid and acetylation, e.g. of sulpha-compounds;

(6) reduction of 'apparent digestibility' of foods;

(7) lowered secretion of enzymes in pancreatic juice;

(8) high retention of nitrogen (large positive nitrogen balance) on feeding a good diet—usually with at first no appreciable increase in weight;

(9) occurrence in hair of altered pigment with chromatographic and spectrographic characteristics resembling those of oxidized normal hair pigment;

(10) increase in fluid in the so-called extracellular 'space'.

Many of the foregoing signs can be seen in human infants and most of them have been observed in young Gambian subjects. *In later childhood, tardiness of development of the sex organs is* frequently seen. It would take too long to examine all these items in detail and references are given to papers which contain data in support of these observations. The change in the hair pigment is, however, selected for fuller consideration.

The colour of the hair changes from the normal black to a reddish-brown in protein undernutrition and there is an alteration of certain properties of the pigment extracted from the reddish-brown hair of the malnourished African, as compared with normal black hair. Details of procedures and an illustration of the difference in behaviour on chromatography have

Protein Undernutrition with Excess of Dietary Carbohydrate

In many countries an infant may be weaned from the breast on to a starchy pap of cassava, arrowroot, yam, sago or plantain, more or less sweetened with sugar and occasionally with additions, one of which might be 'bush tea'—an infusion of leaves from plants some of which contain toxic substances.

Possible sequelae of feeding such diets are neatly illustrated in two sets of experiments made by Lindan (1954) who drew two conclusions from his work: (*a*) that giving identical amounts of the lipogenic diet to rats of different initial body weights and correspondingly different ages reveals that the younger the animals, the better their utilization of food for growth, and the greater their susceptibility to fatty infiltration of the liver; (*b*) that an abundant supply of carbohydrates to young growing animals is a predisposing factor to the development of fatty infiltration Although the diets used in these rat experiments are not identical with those given to human infants, and bearing in mind that the response of the human to lipogenic diets is not necessarily identical with that of rats, from the results of these experiments one may safely infer that the state of nutrition of the infant, the amount of carbohydrate relative to protein in the diet, and the age at which an unbalanced diet is given may determine the amount of fatty infiltration of the liver and the rate at which it develops. The severity of the clinical manifestations might be expected to be related to the degree of and damage due to protein undernutrition but the culminating phase may be a more or less acute event consequent on the development of the fatty liver The extent to which fat accumulation may occur is illustrated in Plate IX, Figure 5; I always wonder how an organ so clogged with fat can function at all.

Apart from the addition of the effects of a more or less completely fatty liver—which will by interfering with protein synthesis exacerbate already existing evidences of protein deficiency—what other features may this form of protein malnutrition present? In the form of severe protein malnutrition, known as kwashiorkor, there is such aggravation of many of the signs of protein malnutrition that the patient becomes seriously

(Nieweg and Arends, 1955) in rats fed on a low protein diet which was, however, high in fat.

I must stress here that often mother's milk has been the main if not the sole food of the Gambian children with signs of protein malnutrition; the signs are often mild and it would seem from the nitrogen metabolism data we have that the infant has adjusted itself to a low plane of protein nutrition. The sort of adjustment in metabolism that might be made is illustrated from some recent work (Sprinson and Rittenberg, 1949) with isotopic nitrogen compounds, in which it was shown that men and rats obtained an appreciable fraction of their nitrogen for protein anabolism from ammonium salts when the level of the protein in the diet was low, but that this source of nitrogen was not used when the protein supply was ample. The cytological changes in, for example, the liver, also appear to reflect the biochemical adjustments to the plane of nutrition and some such adjustment may explain the infiltration of the cytoplasm of the parenchymal cells of the liver with glycogen. This infiltration is easily reversed (Balfour, 1955) when adequate protein is given in the diet, and there is no evidence that the liver cells are permanently damaged or that they die. In the cells of the pancreas of the animal on a low protein diet there is a great reduction in the number of zymogen granules; it seems probable that this is an adaptation to the dietary regime since normal granule production is restored by feeding a balanced ration containing ample protein. Apart from this reduction in granule formation the pancreatic cells appear to be undamaged, even in young animals dying of inanition after being kept since weaning on a diet low in protein (0·5 per cent casein) but adequate in respect of the known accessory food factors. When however the protein poor diet is one consisting entirely of cassava meal (imbalanced with respect to accessory food factors and having a protein of poor biological value of imperfect essential amino-acid balance) the pancreatic cells are irreversibly damaged: the explanation of this phenomenon has not yet been worked out.

work. Loss of protein from the liver is relatively early and large (25 per cent in three days); but in 59 days the liver contributes only 7 per cent to the total lost, plasma 2 per cent, whilst 91 per cent comes from the remaining tissues (Hegsted *et al.*, 1950). Recovery is likewise slow as is shown by the data of Demaeyer and Vanderborght (1954) (see Figure 6) on serum albumin in the course of recovery. There are other evidences of slow recovery. I have suggested (Anon , 1952) that blood albumin might be used as a monitor, but its value as such should be assessed in relation to the fact that the plasma albumin losses may be as little as 2 per cent of the total losses of body protein.

When attempts are made to rear a young child on the family diet, it is not surprising to find signs in the child of the nutrient imbalance characteristic of the diet. Frontali (1954) has clearly shown how deficiencies such as of vitamins A and B_1 and of nicotinic acid and pyridoxine may be dissected clinically from the protein malnutrition syndrome he prefers to call 'starchy food dystrophy'. In a recent lecture at the Institute of Child Health, Great Ormond Street, he said that this syndrome had in the past been widespread throughout Europe. It was, however, notable that he omitted the British Isles from his list of countries in which its occurrence had been reported; it was also remarkable that he did not include signs of rickets, i.e. of vitamin D deficiency, as an accompaniment of the syndrome. I raised these two points at the conclusion of Frontali's lecture and I have since been searching for evidence of the occurrence of protein malnutrition in the British Isles, possibly as starchy food dystrophy might be expected from the extent of 'pap' feeding of infants in the eighteenth and nineteenth centuries, and that it might well have been associated with 'the English disease', rickets. Two relevant statements are still to be found in the account on rickets in Price's *Practice of Medicine* (1946)—'The fault is usually dietetic and not only because of deficiency of vitamin D containing foods but also because excess of carbohydrate promotes more rapid growth than calcification can cope with', and 'The edge of the liver can often be felt at a lower level than normal, partly from displacement and in part from enlargement through fatty change'. It is also interesting that

ill and often dies; dermatosis may be marked and characteristic and different from that of pellagra (Platt, 1953b); the only new feature which might be expected, hepatomegaly, is not always present. In my view, applying in a different way a simile that has already been used in this connection, the low protein—high carbohydrate group of diseases (kwashiorkor, starchy food dystrophy and their congeners) constitute a band in a spectrum of protein undernutrition which merges into other forms, including cases with various degrees of marasmus. Unless some new and peculiar accompaniment of fatty liver is discovered,

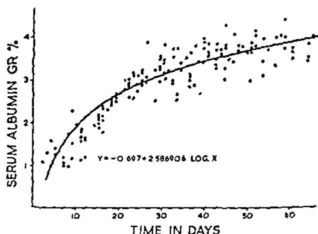


Fig. 6 Showing serum levels in West African children during recovery from protein malnutrition (after Demaeyer and Vanderborght, 1954).

we have no specific evidence of its occurrence except the presence of extra fat in the liver! One fact, however, is beyond dispute, that is the life-saving and remedial value of the administration of good protein. Some of us, appreciating the fact that components of cytoplasm other than protein may be needed for restoration of tissues have given potassium salts in severe cases, and a good balanced diet is to be recommended. There may be a special requirement for extra riboflavin, some of the other members of the *B*-vitamin group, and ascorbic acid.

The fact that it takes many weeks to deplete the body's protein is not always appreciated—at any rate, in experimental

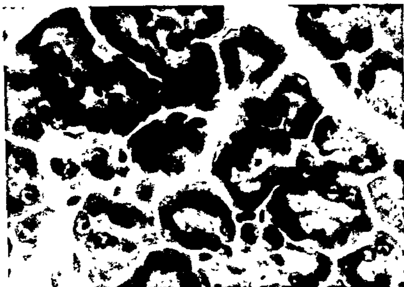


FIG 1. Section of pancreas from weanling rat fed on cassava + 5·5% groundnuts for 30 days The acinar cells are 'lean and hungry', they are somewhat smaller than normal and there is a great reduction in the number of zymogen granules. (Hx & E × 600 approx)

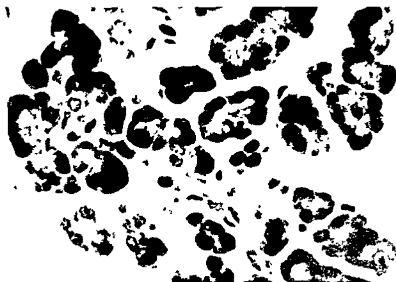


FIG 2 Pancreas from weanling rat fed on cassava for 30 days The acinar cells are extremely small and contain practically no zymogen granules but there is no evidence of irreversible damage to the cells (Hx & E × 600 approx)

200 years ago rickets was associated with the notion of a 'weke liver' and that the earliest seventeenth-century Bills of Mortality for London do not mention rickets but 'we find a curious heading "Livergrown" which in later years is bracketed together with rickets, while after 1634 they are listed separately' (Drummond and Wilbraham, 1939).

Conditioning Factors in Protein Undernutrition

By 'conditioning factors' I mean causes other than dietary ones [illegible]

In thinking about the possible effects of malarial parasites on the protein economy of the body, three conditions persistently come to mind; they are:

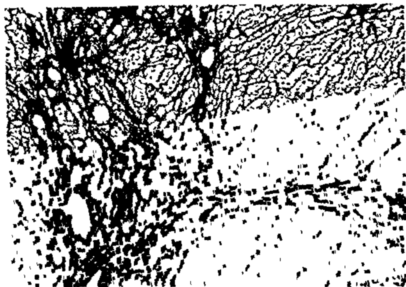
(1) that the effects of a variety of toxic agents are specially manifest when they are exhibited in animals on diets containing sub-optimal amounts of protein; this may have a bearing on the effects of the toxins produced by malarial parasites if, indeed, there are any, on more or less undernourished tissues;

(2) that a prominent feature of many types of injury and of diseases due to zymotic factors is a more or less prolonged loss of nitrogen from the body,

(3) that some features which are common to several states of disease can be included in the medical shock syndrome, one element of which is a disturbance of protein metabolism.

We do not know enough about these phenomena to examine them in detail in a study of the interrelationships of malnutrition and malaria. The possible effects on protein nutrition of malaria infestation may however be summarized: (*a*) reduction of effective protein supplies· the result of anorexia; vomiting and diarrhoea, impaired anabolism—due, for example, to liver damage, losses by proteinuria; abnormal distribution of protein between vascular and other body compartments; and (*b*) increased requirements: the result of feeding the parasite, especially with haemoglobin; excessive protein catabolism; synthesis of the materials of immunity—humoral and cellular—against the parasite; and replacements or repair of damaged tissues.

PLATE VIII



...man liver showing fairly advanced
...the lobular architecture (Silver × 100 approx.)

PLATE VII

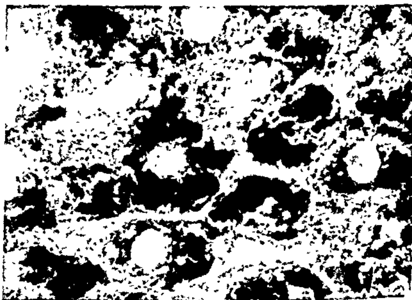


FIG. 3 Liver from adult rat fed on protein-free diet for 5 days. The liver
[illegible]

It has been known for a long time that a lowering of plasma albumin levels is often a feature of malaria. Taylor, Mickelsen and Keys (1949) observed a reduction in serum albumin concentration and in circulating albumin together with a marked rise in alpha globulins after a mild attack of malaria (*Plasmodium vivax*) of short duration, but these authors concluded that there is no valid evidence which relates these changes to the protein deficiency of caloric undernutrition which may accompany malaria and other diseases showing similar changes in serum proteins. Although we have only scanty quantitative data on the effects of malaria infestations on protein supplies and requirements, it would seem that a combination of the various factors enumerated might considerably reduce the supplies of protein available for normal physiological purposes. These effects on the already sub-optimally nourished subject, especially at times when requirements are naturally high—during periods of rapid growth and during child-bearing—may well lead to aggravation of the effects of protein malnutrition, possibly even with fatal consequences. Malaria is, in fact, recognized as being in many countries a main cause of death and is considered to be especially serious in young children and in pregnancy, causing abortion, premature birth of the infant, and sometimes death of the mother. The infant's ability to cope with malarial parasites seems to some extent to depend on adequate feeding at the breast; I have seen parasites disappear from the blood of an infant whose mother has been taken from work in the rice fields, rested and well fed and thereby enabled to feed her infant properly. Neither mother nor infant was given anti-malarial treatment, and it is questionable whether the effect was due to malarial antibodies in the breast milk since they are probably not transmissible to the human infant in the milk.

If the infested infant survives it comes to terms with the malarial parasite and a state of 'premunition' is achieved. The cost of this 'cold war' in terms of nutrients, especially of protein, has not so far as I know been calculated. The estimate would be well worth making; for some years we have suspected that it might be considerable.

In a Gambian village in which some improvements in food



FIG. 5 Section of liver from rat on diet low in protein and choline Fat droplets of moderate size distributed fairly evenly throughout the lobule. (Oil red O × 130)

malaria (Brooke, 1945; Seeler and Ott, 1945) from which it seems that susceptibility, severity and persistence of parasitization might be related to protein deficiency in the bird's dietary.

SOCIAL FACTORS AND PROTEIN MALNUTRITION

In the second World Food Survey, data are given for the proportions of the world's population having different amounts of protein in the diet (Food and Agriculture Organization, 1952). I have offered (Platt, 1955) some grounds for the view that where sophistication of foods has led, chiefly in those populations in which food supplies are especially abundant, to apparently successful artificial feeding of infants with cows' milk, attention may have to be given to the possibility of overfeeding with protein with the occurrence of a relative imbalance of galactose. High levels of protein in a dietary lead to the selection of a diet of high energy content that may well produce excessive overeating with which detrimental effects on health and reduced longevity have been associated.

Undernutrition, coupled with the effects of diseases such as malaria, may on the other hand cause death or life-long debility which, as medical men, we cannot stand by and allow to continue. Immediate increases in food production are likely to occur in the places where they are most needed by attacks on pests and diseases which damage the growing crops or cause losses of crops during storage. Likewise, human needs for food might well be reduced most quickly and cheaply by eliminating or reducing the toll taken by many zymotic diseases by eradication of those diseases. Even where malaria and malnutrition are prevalent, the African villagers expend energy in work at the same rate as healthy Europeans would in similar climatic conditions Their ability to do the same numbers of hours of work at high levels of output is, however, less; but it might well be increased by the eradication of parasites and the improvement of nutrition. Increase of human physical effort—or mechanical aids to production—will be, together with increased and sustained soil fertility, essentials in increasing food production in the future.

Measures successful in improving health of impoverished communities are likely to increase the rate of growth of population

production and feeding were instituted and in another where antimalarial measures were applied, similar improvements were observed (Platt, 1953c). In the former there were reductions in infant mortality, in the occurrence of signs attributable to insufficiency of certain B-vitamins, and of oedema; there were increases in body weight and evidences of improved zest. In the other village there was also a reduction in infant mortality, recession of a number of signs of malnutrition, an all-round improvement in haemoglobin levels, spontaneous transformation from an untidy to a well-kept village, and a reduction in the children of up to 16 years of age of about 50 per cent in the malarial parasite load. The level of haemoglobin content of the blood of the group in the village containing the nursing mothers was extremely low. The records of these experiments have not been fully analysed but it is justifiable to conclude that the amount and extent of protein malnutrition had been reduced in both villages; some of the clinical changes can be attributed to the increases in haemoglobin concentration and I would suggest that one factor in the reduced infant mortality might be in the improvement of lactation.

I found in 1951 that the malarial parasitaemia of Gambian infants given very small doses of chloroquin was suppressed for several days. This observation provided a basis for a feasible trial of suppressive therapy from birth. The results of this, it is hoped, will help in the evaluation of the relative effects of malaria and malnutrition. After one year the significant differences between protected and control groups were, as might be expected, that the former had no parasites, no enlargement of the spleen, significantly fewer palpable livers, and a mean haemoglobin of 2 6g per cent higher than that of the unprotected infants. The unprotected infants tended to put on less weight in a year and more of them had reddish-brown hair; more of the unprotected infants died in the first year. From this series of observations equally impressive results might be expected to follow a combination of protection of the infant and nutritional rehabilitation of the mother.

Supporting evidence on the interrelationship of the state of nutrition and malarial infection is found in studies of bird

it is significant that well-to-do mothers, after having their babies, returned for a time to the 'fatting-house'.

The illustrations of my theme have been drawn mainly from a study of malnutrition in the early years of life when protein metabolism is especially intense. Mothers should also be predominant in the picture because of increased protein metabolism particularly during child-bearing and especially throughout lactation. Modern artificial methods of feeding infants might well lead to excessive feeding with protein and I believe there are good arguments for reverting to or retaining some primitive feeding practices which might, like 'on demand' feeding, prove to be superior to some modern ones. In general, for the prevention of protein undernutrition in the young, more emphasis should be placed on feeding the mother so as to enable her to sustain an ample and prolonged supply of milk.

Finally, may I add that, notwithstanding the paramount importance of proteins in nutrition, for the growth, maintenance and rehabilitation of tissues, other nutrients must be supplied in the diet and a balanced diet of good food must remain the sheet-anchor of treatment—curative and preventive.

REFERENCES

[illegible]

34, 417

DOLE, V P, DAHL, L K, SCHWARTZ, I L, COTZIAS, G. C, THAYSEN, J H and HARRIS, C (1953) *J clin Invest* 32, 185

DRUMMOND, J C and WILBRAHAM, A (1939). *The Englishman's Food*, p 192 Cape, London

FOOD AND AGRICULTURE ORGANIZATION (1952) Second World Food Survey, Rome

FRONTALI, G (1954) *Rep 2nd inter-Afr (CCTA) Conf Nutr, Gambia 1952*, p 102 H M S O, London

HEGSTED, D M, ZAMCHECK, M D, WANG, C. F. and BLACK, M B (1950) In *Plasma Proteins*, ed. Youmans, vol. ii, p 238. Thomas, Illinois

adding, in time, greater weight to what is already recognized to be one of the world's greatest problems. I have discussed (Platt, 1954d) this matter and as a contribution to a solution I have argued the case for starting with the improvement of the health and welfare of mothers and infants. The logic of the case from the medical scientist's point of view should be supported by the evidence I have given you in this lecture.

One crude aspect of this problem was brought home to me in some experiments in my Unit in which it was found that mother rats on 'African diet' ate their offspring. Incredible though it may seem, this observation has a 'human' counterpart; the Australian aboriginal is reported to have practised infanticide and cannibalism, the infant sometimes being fed to older children to make them strong (Platt, 1954a). In primitive communities the infant born 'too soon' after its predecessor is sometimes given a name which indicates that it has taken the birthright (of breast feeding) of its predecessor and the name carries a stigma for the parents of such a child. A similar idea is inherent in the term 'kwashiorkor' which means 'the deposed child' and has now come to be identified with the disease which develops if the elder infant is fed on unsuitable substitutes for its mother's milk. Amongst people on poor subsistence food economies, infanticide of one or both of twins not infrequently occurs and less than fifty years ago mothers of twins were murdered or ostracized. The hazard of life for twins—and their mothers—has been supported by Woodruff (1955) who recently found profound evidences of protein malnutrition in pregnant women, prominent among which was anaemia, and twin pregnancies were four times as common among anaemic as in non-anaemic women delivered in hospital at Ibadan, Nigeria.

Many of the gruesome elements of malnutrition are things of the past· but as a world problem there may be greater risks of catastrophes ahead The remedy for an ill often lies near to the ill—in Nigeria the 'fatting house' was, up to the present century, an institution which turned out brides well equipped for pregnancy. Whether this was merely a fashion for enhanced desirability or had some deeper-seated origin is difficult to say; but

it is significant that well-to-do mothers, after having their babies, returned for a time to the 'fatting-house'.

The illustrations of my theme have been drawn mainly from a study of malnutrition in the early years of life when protein metabolism is especially intense. Mothers should also be predominant in the picture because of increased protein metabolism particularly during child-bearing and especially throughout lactation. Modern artificial methods of feeding infants might well lead to excessive feeding with protein and I believe there are good arguments for reverting to or retaining some primitive feeding practices which might, like 'on demand' feeding, prove to be superior to some modern ones. In general, for the prevention of protein undernutrition in the young, more emphasis should be placed on feeding the mother so as to enable her to sustain an ample and prolonged supply of milk.

Finally, may I add that, notwithstanding the paramount importance of proteins in nutrition, for the growth, maintenance and rehabilitation of tissues, other nutrients must be supplied in the diet and a balanced diet of good food must remain the sheet-anchor of treatment—curative and preventive.

REFERENCES

ANON (1952) *Brit Med J* ii, 821.

BALFOUR, B M (1955) Personal communication

BROOKE, M M. (1945) *Amer J Hyg* 41, 81.

CREERY, R D G and NEILL, D. W (1954) *Lancet*, ii, 110

DEMAEYER, E M and VANDERBORGHT, H (1954) *Ann Soc belge Méd trop* 34, 417

DOLE, V P, DAHL, L K, SCHWARTZ, I L, COTZIAS, G C, THAYSEN, J H and HARRIS, C (1953) *J clin Invest* 32, 185.

DRUMMOND, J C. and WILBRAHAM, A (1939). *The Englishman's Food*, p 192 Cape, London

FOOD AND AGRICULTURE ORGANIZATION (1952) Second World Food Survey, Rome

FRONTALI, G (1954) *Rep 2nd inter-Afr. (CCTA) Conf Nutr, Gambia 1952*, p 102 H M S O, London

HEGSTED, D M, ZAMCHECK, M D, WANG, C F and BLACK, M. B (1950) In *Plasma Proteins*, ed Youmans, vol. ii, p 238 Thomas, Illinois

JEANS, P. C. (1952). *Handbook of Nutrition*, 2nd edn., Am. med. Assn, p. 275 Lewis, London.

LINDAN, O. (1954). *Rep. 2nd inter-Afr. (CCTA) Conf. Nutr, Gambia 1952*, p. 129. H.M.S.O., London

LOWE, K. G., HENDERSON, J. L., PARK, W. W. and MCGREAL, D. A. (1954). *Lancet*, ii, 101.

NAGCHAUDHURI, J. and PLATT, B S. (1954). *Rep. 2nd inter-Afr. (CCTA) Conf. Nutr., Gambia 1952*, p. 215. H.M.S.O , London.

NIEWEG, H. O. and ARENDS, A. (1955). *Rep. 3rd int. Congr. Nutr, Amsterdam 1954. Voeding*, 16, 256

PLATT, B. S. (1953a). *Proc. Vth int Congr. trop. Med. Malaria, Istanbul 1953*, p. 155.

PLATT, B S. (1953b). In *Handbook of Tropical Dermatology*, ed. Simons, 2, 1476. Elsevier, Amsterdam.

PLATT, B. S. (1953c). In *Development of Tropical and Sub-Tropical Countries*, ed. Banks, p. 97. Arnold, London.

[illegible] *TA) Conf. Nutr., Gambia 1952*, p. 153. H.M.S.O., London.

PLATT, B. S. (1954d). *Lancet*, ii, 929.

PLATT, B S (1955). *Brit. med J.* i, 179.

PLATT, B. S. and MONCRIEFF, A A. (1947). *Brit med. Bull.* 5, 177

PRICE, F. W. (1946). *A Textbook of the Practice of Medicine*, 7th edn., ed. Price, [illegible] Oxford University Press, London.

[illegible] *is.* 77, 181.

[illegible] *ol. Chem.* 180, 707.

TAYLOR, H. L., MICKELSON, O and KEYS, A. (1949). *J. clin Invest*, 28, 273.

WALTERS, J H and WATERLOW, J. C. (1954) Fibrosis of the Liver in West African Children. *Spec. Rep. Ser. med Res Coun., Lond*, No 285

WHIPPLE, G H and ROBSCHEIT-ROBBINS, F. S. (1951). *J. exp. Med.* 94, 223

WOODRUFF, A. W. (1955). *Brit med. J* i, 1297.

WORLD HEALTH ORGANIZATION. (1955). 4th Rep. Joint FAO/WHO Expert Committee on Nutrition. *Wld Hlth Org techn. Rep. Ser.* 97.

X

Shock from Burns

J. P. BULL

A SEVERE burn of perhaps one-third of the body surface constitutes a gross injury to an important organ, and it would be unreasonable to expect that all sequelae of the injury could be attributed to a single disturbance. We must rather try to identify the main changes, if possible find their causal relations and from the clinical point of view pay particular attention to the aspects which can be corrected by treatment. As long ago as 1881 Tappeiner noted one of the most fundamental changes following severe burns, i.e. the haemoconcentration resulting from plasma loss. More recent experiments have shown how extensive this loss can be. A burn of half the body surface in animals may cause local fluid loss into the tissues about equal to the plasma volume as was demonstrated in wartime experiments (Rossiter, 1943). More recently the late Dr. Everett Evans and his co-workers in Richmond, Virginia, showed in dogs that loss of plasma was approximately 1 ml./1 per cent body burned/kilogram body weight (Brooks *et al.*, 1951). This fits well with the previous work in England. In man, these local losses are often obvious, the burned hand may swell to twice its normal size, and the loose tissues of the face may be so filled with fluid as to make a person unrecognizable. As in shock due to loss of whole blood, a moderate loss of, say, one-fifth of the circulatory volume can be tolerated without marked symptoms A patient with a burn causing loss of a similar volume of plasma will have a correspondingly raised haematocrit but will recover with treatment by oral fluids only and may never show signs of severe circulatory

disturbance. With more extensive loss, the classical picture described by Grant and Reeve (1951) as 'cold hypotension' can readily ensue, and the onset of these symptoms and signs may be rather insidious since the fluid loss continues over a period of hours, about half the acute loss occurring within eight hours and the remainder within the subsequent twenty-four hours. Perhaps on account of the special type of injury involved, a large area of damage to the skin, the detailed circulatory changes in burns shock are often different from those following simple haemorrhage. Using cardiac catheterization, the Bellevue group in New York showed that even when great loss of circulatory volume had occurred, the peripheral blood pressure might still be normal (Richards, 1944). Constriction of the veins and raised peripheral resistance are prominent features in severe burns. When the blood pressure finally gives way the situation is often desperate and may be irreversible. Similar findings showing this relatively delayed fall of blood pressure were reported from detailed studies made in Glasgow during the war (Colebrook *et al.*, 1945).

Capillary Permeability

The damage to capillaries in a burn causes them to become permeable to plasma protein and the greatly increased flow of lymph from a burned area contains a proportion of all the normal plasma proteins, even including the large molecules such as fibrinogen. For this reason the fluid in blisters may clot spontaneously. The total protein level is not much less than that in the circulation. We have found values commonly around 5 gms./100 ml. so that the loss of fluid from the circulation can be taken to be substantially that of whole plasma. Included with the protein is the normal complement of electrolytes. If in therapy a plasma substitute such as dextran or polyvinyl pyrollidone is given, these substances will also appear in oedema fluid.

It seems very probable that a chemical mediator is responsible for the local increase in capillary permeability. Histamine is capable of producing many of the changes observed and can be found to be increased in the circulation following burns (Barsoum

and Gaddum, 1936). On the other hand the levels found do not correspond closely to the severity of burning, and experiments with anti-histamines (Sevitt, 1949) have shown that these compounds have little effect on animal burns. We have confirmed this upon experimental burns in man and have found that the burns of people receiving systemic anti-histamine behave similarly to those of persons treated with inactive tablets (Sevitt *et al.*, 1952). Leukotaxine is another possible agent and has been shown by Cullumbine and others (1947) to produce on local injection the oedema and haemoconcentration found in burning. Another line of inquiry has been the possible release of enzymes by burned tissues. A proteinase has been described by Beloff and Peters (1944) which is resistant to heating to 70° C. and which appears to leave the tissues after burning. Cope's group in Boston also demonstrated a peptidase activity in lymph and serum after experimental burns (Zamecnik *et al*, 1945). A further link in this possible chain of causation is Cullumbine's demonstration that the proteinase described by Beloff and Peters can cause liberation of leukotaxine by its action on fibrinogen. The limitation of oedema by cooling has also been attributed to slowing of some enzymic process but Courtice (1946) showed that the effect was consistent with the associated reduction of blood flow.

Electrolyte and Fluid Balance

Further to the exchange of electrolytes and fluids between the circulation and the extravascular space resulting from increased capillary permeability, there is the exchange of potassium and sodium between the damaged cells and the extravascular space This exchange can be demonstrated experimentally but in comparison with the gross amounts involved in the leak from the circulation, the quantities of potassium lost by the damaged skin and sodium taken up by it are rather small (Moore *et al.*, 1948). It is exceptional in reasonably well-treated cases for there to be any considerable rise in serum potassium, though such rises can be demonstrated in severe animal burns which are untreated, and should treatment of human cases be delayed and renal 'shut down' occur, the danger of an accumulation of

potassium becomes serious. A feature of burns treated by orthodox methods is that the serum sodium level tends to fall during the shock period. *Magee and Spector (1951) showed similar* changes in rabbits produced by acute anhydraemia induced either by burning or by subcutaneous hypertonic sugar. They interpret their results as being due to increase in extracellular *fluid volume and this may easily be a factor in our burns* patients, though we have considerable difficulty in obtaining normal or high levels of serum sodium even after giving large amounts of sodium (Bull and England, 1954). These experiments were part of a detailed study of the electrolyte and water balances of a series of severely burned patients, and our findings confirm in general those of Professor Francis Moore who was a pioneer in this field. There is an initial period of marked sodium retention, at the same time as a fairly brisk potassium excretion (Figure 1). The subsequent reversal of the sodium balance coincides commonly with a diminution of extravascular fluid. The swing of nitrogen balance is similar to that found after other forms of severe trauma, i.e. a period of marked negative balance at about 3–8 days followed by a positive balance during convalescence.

[illegible] after burn-

[illegible]

500 ml. per day can fairly easily be obtained even in the very severely burned if treatment is started early but administration of greatly increased amounts of fluid, or for that matter, colloid or sodium, produces very little further flow. This is particularly true of large burns. Many small burns do respond by excreting extra amounts of fluid given and a corollary is that if the urine output is good then fluid management is almost certainly satisfactory; but we have not had these high flows with the larger burns and have rather come to accept an oliguria of about 500 ml./day as a reasonable optimum. No great urea retention occurs with such flows, and any slight rises are subsequently quickly corrected. Our finding of good renal concentration in this series of cases did not suggest that tubular necrosis was present.

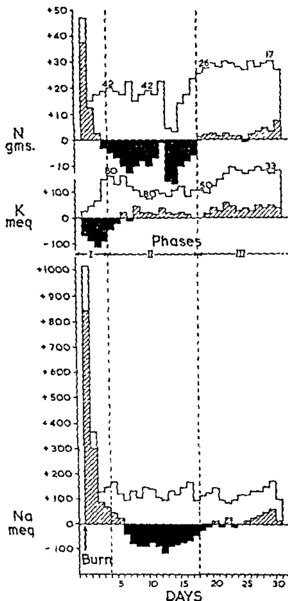


FIG 1 Typical nitrogen, potassium and sodium balances in severe burns. (From Moore and Ball, 1952.)

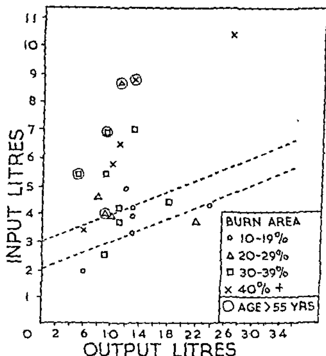


FIG 2 Intake of non-colloid fluid and output of urine in first 48 hours after burn (From Bull and England, 1954)

ELECTROLYTE BALANCE

This general disturbance of electrolyte metabolism and excretion reminds one of the effects of adrenocortical hormones, and there is evidence that these hormones are concerned in the response to burning. It is probable that adrenaline is released initially and this stimulates the production of ACTH which itself causes release of adrenocortical hormones. The excretion of corticoid and 17-ketosteroid substances is raised in severe burns by an amount proportional to the size of the burn. Evans and Butterfield (1951) have shown that administration of 1 gm./day of Compound F leads to a very closely similar metabolic picture to that following burning, particularly in relation to sodium and potassium balance and the depression of circulating eosinophils. The water balance was not quite so similar,

and it is possible that in burns some water retention mediated by the antidiuretic hormone also occurs.

Another approach to this problem of the mechanism of fluid and salt exchange which is of great clinical interest is that on the lines of the work of Borst and de Vries (1950). These workers postulate that renal excretion of water and salt is determined in some way by the circulatory volume, the link being provided perhaps by a flow receptor. We have not been able to confirm this directly in our burns cases as yet, since only recently has it been feasible to obtain accurate serial blood-volumes upon these cases. Our less severe shock cases certainly appear to respond to extra colloid infusion by improved and virtually normal excretion of water and salt, but the more severe cases, and these are the ones in which the metabolic disturbances are most marked, still show the phase of sodium and water retention which I have described. We hope that further studies in progress will throw more light on the role of circulatory volume in these cases also.

Red Cell Loss

One of the complications in such studies upon severely burned patients is that as well as the water, salt and colloid changes already described, there is a loss of red cells. My colleague, Dr. Topley, has been studying this problem now for some time, and finds that some of the patients with large areas of full thickness loss may lose in the first 48 hours after burning 40 or 50 per cent of their circulating red cells (Topley, 1954). This early loss seems to be only of considerable importance in burns of more than 30 per cent of the body area, but it is not the same from case to case, and at present we are trying to devise methods for its recognition and appropriate therapy. In such cases the haematocrit, which is usually the best guide to haemoconcentration and changes in volume following burns, becomes unreliable and leads one to underestimate the transfusion requirements of the patient. We do not yet know the mechanism of this loss of red cells: doubtless some are haemolysed by being directly heated; others may be trapped in vessels which become sealed off from the circulation; though the main

factor is probably intravascular haemolysis. The immediate loss involves only the patient's own cells, but subsequently we find a continuing loss of red cells which involves both the patient's and transfused cells. Again, this continued loss seems *largely to be haemolytic in type, though changes in haemo*globin metabolism are also probably involved.

PRINCIPLES OF TREATMENT

We can recapitulate the clinical implications of our present knowledge of the early changes after a severe burn by considering the treatment of a typical patient. A child has perhaps fallen into a bath of hot water and received burns of limbs and trunk. *It is important at this stage to anticipate the development* of shock and to do this efficiently we must know what percentage of the body surface is burned. Mapping out the area on a shock chart, we calculate that this burn involved 35 per cent of the body surface. We know *from past experience* that burns of 10 per cent or more can be expected to need plasma or similar transfusion, so that the immediate treatment is to set up an intravenous drip. Since, as I have mentioned, venous constriction is a very common feature of such cases, we usually find that it is necessary to insert a cannula. At the same time a specimen for a haematocrit estimation is taken, but already plasma is running in. The specimen of blood is taken by the doctor to a side room where the capillary haematocrit tube is filled and spun in a centrifuge. After a few minutes this will be ready and an immediate assessment of the appropriate rate of plasma transfusion made. The aim of therapy is to maintain a satisfactory circulatory volume with the minimum of disturbance. We try to regain a normal haematocrit value *within* an hour or two and then to maintain this value by continuing plasma transfusion at a *slower* rate. Subsequent haematocrit specimens can be taken from capillary blood obtained by needle prick *from* an area *with* active *circulation A chart* of the patient's progress and treatment is commenced and typically shows that most of the plasma is given within 24 hours, half of it within 6–8 hours; the detailed changes of haematocrit can be *seen to result from changes in speed of transfusion.* It is our

practice for larger burns to give whole blood transfusion also, either during the shock phase or immediately after. As mentioned previously, burns somewhat greater than the one we have described commonly lose considerable quantities of red cells during this early period, and we replace this loss by blood transfusion. As an alternative to measures of haemoconcentration such as the haematocrit and haemoglobin, some people prefer to use blood-pressure measurements. With experience this may be satisfactory, but our impression confirms previous studies that fall in blood pressure is a relatively late sign and that the earlier sign of haemoconcentration is a more sensitive guide. Another objection to the use of blood pressure is that it is frequently physically difficult to measure since many severe burns involve areas on the limbs most convenient for blood-pressure determinations. Apart from haematocrit we will be watching the changes in skin temperature, the urine output and symptoms such as restlessness and thirst. While plasma is being given intravenously, frequent small quantities of fluids are given by mouth, sufficient to supply the normal requirements for urinary and extrarenal losses with an extra allowance if there is any fever. For children, total oral fluids of 1-2 litres per day are satisfactory; in adults 3-4 litres may be necessary. Whether extra salt should be given at this time is doubtful since reconstituted plasma and bottled blood, which both provide a protein level similar to that in burns exudate, also contain rather more sodium (about 165 m.eq. per litre) than is normally present in extracellular fluid. If such material is being transfused to provide for exudate losses, no great electrolyte disturbances follow the use of either no added oral sodium or a moderate addition. Review of cases treated by these methods shows that the total amount of plasma, or its equivalent, required is approximately related to the size of burn. Each 10 per cent surface area in an adult requires about 1 litre of plasma and several formulae have been devised to assist in calculating the fluid requirements for different sizes of burn (Cope and Moore, 1947; Wallace, 1951; Evans *et al.*, 1952). Rigid adherence to a formula probably results in unsatisfactory treatment of some cases, since surface area is not a completely adequate measure of fluid requirement.

We therefore prefer to take each case on its merits and to use the formula only as a secondary check in doubtful cases.

ASSESSMENT OF RESULTS

We have tried to assess the results of our treatment of burns by investigating the mortality, the healing time, and the residual deformity. In assessing results of treatment of the shock stage mortality is particularly appropriate, and we find that it lends itself to a useful standard of success. If we consider all the 3,000 or so patients whom we have treated at the Birmingham Unit

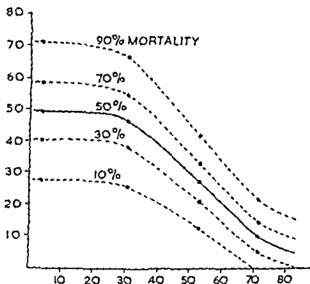


FIG. 3. Equal mortality contours for various combinations of age and area burned. (From Bull and Fisher, 1954.) (Ordinate: percentage surface area burned. Abscissa: Age in years.)

in the last ten years, we find that mortality increases with two characteristics of the patients. The larger the surface area burned, the greater is the mortality; similarly the older the patient, the greater again is the mortality. By considering separate age groups, we have fitted Probit lines for the percentage mortality experienced with different surface areas burned (Bull and Squire, 1949; Bull and Fisher, 1954). From these calculations we can derive the 50 per cent lethal area of burning in relation

to age (Figure 3). In children and young adults a 50 per cent surface area burn has about an even chance of living or dying; in the aged, on the other hand, a 10 per cent burn carries this 50 per cent risk. Derived from these curves we can prepare a grid from which the probable mortality for any series of burns cases can be estimated (Figure 4). This provides a standard for comparing the results of treatment from year to year and from centre to centre, and it also gives a basis for the investigation of the effects of changes in treatment. As an example of this we have been able to use this method to assess the value of dextran as an alternative to plasma in the treatment of burns shock. Our conclusion has been that the use of dextran in moderate amounts, up to the approximate normal value of the patient's plasma volume gives results equivalent to those in cases receiving plasma only.

A feature of this analysis of mortality is that our results in [illegible]

mortality results and the refinements which we have introduced since have not greatly affected the results. A change which has occurred is that many of the fatal cases now survive longer, and perhaps this prolongation of the period of survival is attributable to improvements in treatment Considering these fatal cases in more detail, we see that patients who die in the first two days are mostly those with very severe burns, whereas those dying after the first week include the cases which had about even chances of survival on the basis of our mortality study. Looking at the diagnosis of the causes of death in these burns, we see that apart from the group of early deaths in the very severely burned or the very aged, the common cause of death is infection, often taking the form of pneumonia or septicaemia. Another small group of deaths occurs primarily from intercurrent disease, since many of our elderly patients are already suffering from serious illness, and may indeed burn themselves because of being disabled or perhaps bed-ridden.

% Body Area Burned	Age—Years 0–4	5–9	10–14	15–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–84
78 or more	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
73–77	9	·9	9	·9	·9	1	1	1	1	1	1	1	1	1	1	1	1
68–72	9	9	·9	·9	·9	9	·9	1	1	1	1	1	1	1	1	1	1
63–67	8	8	·8	8	·9	9	·9	9	1	1	1	1	1	1	1	1	1
58–62	7	7	7	7	8	8	·8	9	1	1	1	1	1	1	1	1	1
53–57	6	·6	6	6	·7	·7	·8	8	9	1	1	1	1	1	1	1	1
48–52	·5	5	5	5	·6	·6	7	7	8	·9	1	1	1	1	1	1	1
43–47	4	·4	4	4	·4	·5	·5	6	7	·8	·9	1	1	1	1	1	1
38–42	·3	3	3	3	·3	4	4	5	·6	·7	·9	9	1	1	1	1	1
33–37	2	·2	2	2	2	·3	·3	·4	·5	·6	8	·9	·9	1	1	1	1
28–32	·1	·1	1	1	·2	·2	·2	·3	·4	·5	·6	·8	9	1	1	1	1
23–27	1	·1	·1	·1	1	·1	·1	2	2	·3	4	6	8	·9	1	1	1
18–22	0	0	0	0	0	0	·1	·1	·1	·2	·3	·4	·6	·8	9	1	1
13–17	0	0	0	0	0	0	0	0	·1	·1	·2	·3	·4	·6	·7	8	9
8–12	0	0	0	0	0	0	0	0	0	0	·1	·1	·2	·4	·5	·6	·7
3–7	0	0	0	0	0	0	0	0	0	0	0	0	·1	2	·3	·4	·5
0–2	0	0	0	0	0	0	0	0	0	0	0	0	0	·1	·1	·2	·3

FIG. 4. Grid of Approximate Mortality Probabilities for Various Combinations of Age and Area.
(From Bull and Fisher, 1954.)

Further Developments

The increasing survival of severely burned patients beyond the shock stage draws attention to the importance of the changes subsequent to the acute fluid disturbances which we have so far discussed. We are only beginning to understand some part of these changes. Cope and others (1953) for instance, have recently shown that the metabolic rate may be raised 30–60 per cent after severe burning. They find that this raised B.M.R. is not apparently due to thyroid activity. These results are reminiscent of some earlier work by Muus and Hardenbergh (1944) who were trying to find toxins in the lymph of burned animals. This they were investigating by its action on rat liver slices. Instead of the expected toxic effects they found a raised oxygen consumption. It is possible that some such metabolic stimulant plays a part in the generally increased metabolic rate after burns. As already mentioned, we know that red cell destruction continues so long as any considerable area of whole thickness loss remains. Similarly protein and possibly other important losses occur from the raw surface, so depleting the body's metabolism. Our studies of the electrophoretic picture of the plasma proteins confirm earlier work in showing a marked reduction in circulating albumin and an increase in globulin; these changes are most marked 10–14 days after burning. The rise of γ-globulin can be so extreme that the total serum protein may remain only slightly lowered, though its constitution and colloid osmotic pressure are grossly abnormal. During this stage also infection of the raw area is common, and toxins from the infected surface may easily be playing a part in the common picture of continued illness. It is very striking how such a case improves when the skin is either healed spontaneously or has been successfully grafted. This clinical observation leads naturally to an attempt to excise and graft burns as early as possible after the injury. Until recently we limited this procedure to small full-thickness burns such as occur among workers with hot metals, and we found that immediately grafted burns healed extremely well and more rapidly than when similar cases were grafted at the usual period of slough separation, about the second or third week (Ross, 1950; London, 1953). Recently our

increasing knowledge of the amounts of transfusion required both in burns shock and in grafting operations, based on Dr. Topley's studies, has emboldened us to attempt the immediate excision and grafting of the burns of shock cases. It is too early to assess results as yet, but the method promises to eliminate many of the most troublesome sequelae of severe burns by virtually removing the burn itself and converting the open wound into a closed wound. If the method is successful, it should lead to greatly increased understanding of the physiological and pathological disturbances of burning injuries, as well as, we hope, to improvement in the results of treatment.

REFERENCES

BARSOUM, G. S. and GADDUM, J. H. (1936). *Clin. Sci.* **2**, 357.

BELOFF, A. and PETERS, R. A. (1944) *J Physiol.* **103**, 2P.

BORST, J. G G. and DE VRIES, L A. (1950). *Lancet*, ii, 1.

BROOKS, J. W., ROBINETT, P., LARGEN, T. L. and EVANS, E. L (1951). *Surg. Gynaec. Obstet* **93**, 543.

BULL, J. P. and ENGLAND, N. W. J. (1954) *Lancet*, ii, 9

BULL, J P. and FISHER, A. J. (1954). *Ann. Surg.* **139**, 269

BULL, J. P. and SQUIRE, J. R. (1949) *Ann Surg* **130**, 160.

COLEBROOK, L., GIBSON, T., TODD, J. P, CLARK, A. M., BROWN, A. and ANDERSON, A B. (1945). Med Res. Council Spec. Rep Ser. Lond No. 249.

COPE, O. and MOORE, F. D (1947). *Ann. Surg* **126**, 1010

COPE, O., NARDI, G. L, QUIJANO, M, ROVIT, R L., STANBURY, J B. and WIGHT, A (1953). *Ann Surg.* **137**, 165

COURTICE, F. C. (1946) *J. Physiol* **104**, 321.

CULLUMBINE, H, McDONALD, F. and SIMPSON, M A. (1947). *J. Path. Bact.* **59**, 467.

EVANS, E. I. and BUTTERFIELD, W. J H. (1951) *Ann Surg* **134**, 588.

EVANS, E. I., PURNELL, O J., ROBINETT, P W., BATCHELOR, A. and MARTIN, M (1952) *Ann Surg* **135**, 804.

GRANT, R T and REEVE, E B. (1951) Med. Res. Council Spec. Rep Ser. Lond. No. 277.

LONDON, P. S (1953). *Brit. J. Surg.* **40**, 293.

MAGEE, P. N and SPECTOR, W G. (1951). *Proc roy. Soc*, B, **139**, 584

MOORE, F. D and BALL, M R (1952) *The Metabolic Response to Surgery*, Springfield, Illinois.

MOORE, F. D , EVANS, R. D and BALL, M. R. (1948). *Ann. Surg.* **128**, 266.
[illegible]
ROSSITER, R. J (1943) *Bull. War Med.* **4**, 181.
SEVITT, S. (1949) *Brit. J. exp Path.* **30**, 540.
SEVITT, S., BULL, J. P., CRUICKSHANK, C N. D., JACKSON, D. MACG. and LOWBURY, E. J. L (1952) *Brit. med. J.* **ii**, 57.
TAPPEINER, H. (1881). *Zbl. f. med. Wiss.* **19**, 385.
TOPLEY, E. (1954). *Proc. roy Soc. Med* **47**, 230.
WALLACE, A B. (1951) *Lancet*, **i**, 501.
ZAMECNIK, P. C., STEPHENSON, M. L. and COPE, O. (1945). *J biol. Chem.* **158**, 135.

XI

The Electrolyte and Metabolic Response to Trauma

G. M. WILSON

THE infliction of an injury immediately sets in motion a train of events which have as their purpose the healing of the wound and the restoration of health. The changes that occur are not confined to the immediate vicinity of the wound, for local repair is associated with a more general reaction of the body. Cuthbertson in 1932 first drew attention to the considerable breakdown of protein that occurred after a fracture of bone and since then the further studies of Cuthbertson (1942, 1954) and of Howard (1945) and Moore (1953) have added greatly to our knowledge. The nature and significance of these general bodily changes in convalescence forms the substance of this lecture.

In order to acquire a detailed picture of the biochemical changes that follow the receipt of an injury several methods of study are required. The intake and output of substances from the body may be measured by the familiar metabolic balance technique (Moore and Ball, 1952). At the same time the concentration of substances in the body fluids, particularly the plasma, may be investigated. These methods are limited to the demonstration of changes and afford no information regarding the initial total body composition. The metabolic response to trauma is influenced profoundly by at least two factors, namely the nutritional state of the patient and the nature and severity of the inflicted injury. The well-nourished, healthy adult and the patient depleted by disease show conspicuously different patterns of response after injuries of equal severity.

A detailed study of the general bodily reaction accordingly requires a knowledge both of the initial body composition and of the changes that are subsequently superimposed on this background.

Recent Methods of Study

It is only within recent years that it has been possible to attempt measurements of the amounts of water, fat, sodium, potassium and chloride within the living human body. This has been largely due to the ready availability of suitable isotopes. The principle of the method is simple and has been fully described by Moore and his associates (Edelman *et al.*, 1952a). A known amount of the isotope is added to the body and the quantity excreted during the establishment of equilibrium of distribution is measured. The dilution of the isotope in the corresponding natural element in the body is measured and the total amount of the element with which the isotope has exchanged is calculated. For example, in the case of sodium, the isotope ^{24}Na with a half life of 15 hours may be used. An accurately measured amount of the radioisotope is introduced intravenously either from a calibrated syringe or burette. After a suitable interval, usually about 24 hours, a sample of blood is withdrawn. Then·

$$\text{Total exchangeable sodium in m.eq.} = \frac{\text{Radiosodium injected—radiosodium excreted}}{\text{Radiosodium per m.eq of sodium in plasma}}$$

Similar estimations of the amounts of potassium and chloride in the body may be made with radioisotopes

The same principle is utilized in the measurement of total body water with deuterium oxide. The deuterium exchanges rapidly with the hydrogen of water but is only incorporated to an insignificant extent in organic molecules during the two to three hours required for the measurement in healthy subjects (Schloerb *et al.*, 1950). In this manner considerable information has been gathered about the amount of water in the human body at different ages and in the two sexes (Edelman *et al*, 1952b).

There are several limitations to isotope dilution measurements. The attainment of an equilibrium of distribution of an

isotope is difficult in an essentially dynamic system divided into several compartments with different rates of exchange (Burch *et al.*, 1953). The total exchangeable mass of the element is not necessarily the total amount in the body. The sodium isotopes do not measure a large proportion of the sodium in bone (Davies *et al.*, 1952; Edelman *et al.*, 1954; Miller *et al.*, 1954) though it is improbable that this non-exchangeable sodium is of any importance from the point of view of the rapid metabolic changes after surgery (Bauer, 1954). The potassium in red cells exchanges slowly and is not all included in measurements of exchangeable potassium (Corsa *et al.*, 1950); here again the red cell potassium is not of great metabolic significance. These and other associated problems are being extensively explored, but in spite of the difficulties there can be little doubt that these methods offer the most reliable picture of body composition at present available.

Certain additional information may be deduced from measurements of total body water and exchangeable potassium. *When the water content of the body is known, the fat content* may be calculated on the assumption that lean tissue contains about 73 per cent of water (Rathbun and Pace, 1945).

$$\%\ \text{fat} = 100 - \frac{\%\ \text{water}}{0{\cdot}73}$$

Clearly the assumption regarding the extent of hydration of lean tissue is not justified in many pathological conditions and in the immediate post-operative period. On the other hand in the majority of cases these calculations, though not precise, offer a first approximation of changes in body fat in the presence of disease. The problems associated with calculations of this nature have recently been fully reviewed (Moore *et al.*, 1952; Keys and Brozek, 1953).

The bulk of potassium is in the cellular mass of the body, mostly in the muscles—only about 2 per cent is in the extracellular fluid. Apart from periods of acute stress, measurements of exchangeable potassium reflect changes in the cellular mass of the body. In the healthy female in comparison with the male, there is more fat and less muscle. Fat is anhydrous and much

of the potassium resides in muscles. Accordingly, it is found that the percentage of water and the exchangeable potassium are both less in the female. The difference in relative muscle mass between the male and female is also reflected in a consideration of the ratio of exchangeable potassium and sodium. In the male this is commonly above, and in the female, below unity. In any form of wasting disease the body loses potassium and the ratio consistently falls (Moore *et al.*, 1954).

The isotope dilution method is not only of value in ascertaining the body composition before surgery. Interval determinations may be made while the patient is in hospital and the changes revealed in this manner usually are in good agreement with those shown by metabolic balance techniques (Wilson *et al.*, 1954a). Furthermore, the observations may be extended far out into convalescence after the patient has left hospital when balance methods are no longer applicable. On the other hand, isotope dilution studies cannot be readily repeated more often than once a week so that the immediate changes after trauma are best followed by metabolic balance techniques. It is important to emphasize the advantages that accrue from a combination of the measurements of body composition and of metabolic balance. The latter only affords a limited picture of the biological changes in progress and even in the best conditions available for the study of surgical patients is liable to considerable error. On the other hand, the initial body composition measurements reveal the background on which these changes are imposed and the later measurements can be used as a check of the cumulative changes shown by the balance studies. In this way it is possible to elucidate the rôles played by previous disease and by trauma and to show how changes in body composition alter the response to injury.

The Physiological Response to Injury

The physiological response to an injury in man is best studied in an adult of normal body composition who receives a major surgical operation. It is important that the condition for which the operation is carried out should not have impaired his nutritional state. In such circumstances observations can be

made preoperatively and the whole sequence of events followed. The assessment of the severity of the injury inflicted by a surgical operation is clearly difficult. A loose semi-quantitative scale has been employed (Moore and Ball, 1952). A third degree burn of 25 per cent or more of the body surface is at the top with scale 10. Multiple severe wounds and extensive multi- visceral operations are scale 9 to 7. Scale 5 is represented by such operations as gastrectomy. Appendicectomy and repair of a hernia may be about scale 3 and so on down to scale 1 which may be represented by simple ligation of a vein.

We shall consider in the first instance the metabolic response to a fairly severe injury, about scale 7, in an adult male in good nutritional condition.

Nitrogen balance. In a healthy adult the mean daily intake and output of nitrogen is equal and is about 12 gm. per day. Most of the body nitrogen exists as protein and clearly if lean tissue is being added to the body the patient will show a positive nitrogen balance; conversely if protein is being broken down the patient will show a negative nitrogen balance. If a person is starved the loss of nitrogen is initially about 10 gm. a day but settles down to a rate of about 7 gm. a day after the first 12 days (Benedict, 1915). After an injury of the severity that we are discussing the nitrogen intake for the first day or two is negligible but the excretion rate is unduly high, commonly about 12–15 gm. a day. It is this excessive excretion of nitrogen that characterizes the first phase of the metabolic response to trauma. It represents a breakdown of protein in the body. This accelerated catabolism of protein cannot be prevented by ensuring a high protein intake immediately after the receipt of an injury. Nitrogen taken at this stage is in general only excreted and there is accordingly a negative nitrogen balance which cannot be corrected by any dietetic measures. This stage of accelerated catabolism of protein persists for a few days—perhaps about five days in the type of injury being considered. Thereafter the rate of excretion is reduced and the intake is increased, so gradually balance is achieved. This stage clearly represents the 'turn of the tide'. The rapid ebb of nitrogen from the body is

checked and after a few more days the patient's appetite returns and the body is ready to receive an inflow of nitrogen. Provided an adequate diet with respect to protein and calories is given he then passes into positive nitrogen balance and the lost lean tissue is reconstituted. This restoration of body protein proceeds for several weeks depending on the extent of the original breakdown of lean tissue.

Potassium metabolism. There is a conspicuous loss of potassium on the day of injury and a negative balance usually persists for two or three days, though not for so long as the negative nitrogen balance. In lean tissue there is a fairly constant ratio of about 2·5–3·5 m.eq. of potassium per gram of nitrogen (K : N ratio) (Hastings, 1941). The potassium loss after injury is more than would be predicted on the basis of the nitrogen loss and the K : N ratio is high, usually over 5 m.eq. per gram. As convalescence progresses positive potassium balance is soon achieved and the potassium lost in excess of nitrogen is rapidly restored. Thereafter there is a slower retention of potassium in proportion to the lean tissue restored with a K : N ratio of about 3 m.eq. per gram. There is often relatively little change in the serum potassium concentration but after a severe injury a rise may occur at the time of rapid potassium excretion and a subsequent fall to the preoperative value during the period of potassium retention.

Sodium metabolism. After injury urinary excretion of sodium is extremely low and the amount lost in the sweat and faeces is normally slight. The net balance effect depends largely on the amount of sodium given intravenously or by mouth. Provided that there are no undue extrarenal losses sodium given in the early stage is retained in the body. Usually after a few days any excess sodium is excreted in the urine, but the period of sodium retention is very variable and may be prolonged after a severe injury. If there are large losses through exudate from a wound or by drainage from the alimentary canal a negative balance may develop. The usual features after injury are, however, a stage of sodium retention followed by excretion. A fall in serum sodium concentration commonly occurs after operation. Paradoxically, it is frequently observed that the

serum sodium concentration decreases during the period of sodium retention and rises during the sodium diuresis.

Water metabolism. Immediately after operation there is oliguria and water retention. This water retention develops irrespective of the amount of sodium given and cannot generally be controlled by sodium restriction. If a great excess of water is given by any route a dangerous dilution of body contents occurs (Wynn and Rob, 1954). After two or three days there is normally a diuresis of any excess water taken during the early stage.

Body weight and fat metabolism. There is a rapid drop in weight after an extensive injury and this proceeds for several days. It is greater than can be accounted for in terms of tissue excised and protein catabolism. In these calculations it is assumed that a gram of nitrogen represents 30 gm. of wet lean tissue (Moore and Ball, 1952). The weight loss that cannot be accounted for in these balance studies is presumably due to fat oxidation in the early stage. Serial observations made with deuterium oxide have also shown a reduction in body fat and have confirmed that rapid fat oxidation is a prominent feature of the response to severe injury (Edelman *et al.*, 1952c). Positive nitrogen balances later in convalescence, on the other hand, usually account fully for weight changes, suggesting that replacement of fat does not occur until much later—an interpretation substantiated by clinical observations.

Adrenocortical activity. Immediately after injury there is an increase in the excretion rate of the 17 hydroxycorticoids in the urine. This is usually maximal on the day of injury and the succeeding day and thereafter declines to the pre-trauma level within about seven days. Such a general pattern has been described by several groups of workers using different methods for the determination of urinary corticoids (Venning *et al.*, 1944; Norymberski *et al.*, 1953, Moore *et al.*, 1955). By contrast there is often little change in the excretion of urinary ketosteroids which may show a small rise on the day of injury but thereafter little change (Bennet and Moore, 1951). More recently the blood levels of 17 hydroxycorticoids have been studied after severe injury. In those with uncomplicated courses high levels

have been recorded only for the first two days (Franksson *et al.*, 1954). The blood levels apparently fall before the urinary excretion returns to the normal rate.

The behaviour of the blood eosinophils has been frequently investigated after injury and surgical operations. It is well recognized that the count rapidly falls to zero at which level it remains for a day or two and then climbs to the preoperative level or higher—an 'overshoot' of this nature is frequently observed a week or so after injury. Frequently these fluctuations in the eosinophil count correlate closely with the alterations in 17 hydroxycorticoid excretion. However, it is now widely recognized that the eosinophil count is an unreliable index of adrenocortical activity. Indeed, after the operation of bilateral adrenalectomy when the patient is kept on a fixed daily intake of cortisone the characteristic changes in eosinophil count are seen with a drop to zero and subsequent temporary rise to a high level Clearly in these circumstances the eosinophil count does not reflect a change in the supply of adrenal cortical hormone

The Influence of the Nature and Severity of the Trauma

The typical metabolic response has been described in a well-nourished adult receiving a major surgical operation involving only soft tissues. The response is considerably affected by the extent of a soft tissue injury.

Minor soft tissue injuries. Operations such as repair of an inguinal hernia or uncomplicated appendicectomy elicit a response which is smaller and of shorter duration than that seen in major trauma. Indeed, with the simplest operations the phase of accelerated catabolism is difficult to detect and there are often few features that cannot be explained simply by the enforced reduction of diet Definite weight loss is demonstrable and there is an elevation in the urinary 17 hydroxycorticoid excretion not usually lasting more than a day. Changes in serum electrolytes are not conspicuous but if frequent serial readings are made a drop of about 1–3 m eq./l. may be demonstrated in the sodium and chloride determinations.

Major soft tissue injuries. In these cases if the patient before injury was in good health there is a profound metabolic response. The duration and extent of protein catabolism is increased and a considerable quantity of fat is burnt. A prolonged period of sodium retention is frequently noted. The urinary excretion of 17 hydroxycorticoids is increased over a longer period. It is to be noted that this type of reaction also occurs after relatively minor trauma if sepsis subsequently develops, for example appendicectomy complicated by the development of a pelvic abscess.

Bone fractures. Cuthbertson in his early studies (1932) showed that there was a profound nitrogen loss after fracture of long bones. This has been confirmed in later studies. In injuries of this type there is a prolonged phase of protein catabolism despite adequate intakes of nitrogen and calories. By contrast the 17 hydroxycorticoid excretion is similar to that seen in soft tissue injury and only lasts a few days. The excessive protein catabolism may continue for a month—far longer than there is any evidence of increased corticoid excretion. In one series of cases an interesting contrast has been made between midshaft long bone fracture on the one hand and intracapsular fracture of the neck of the femur on the other. The former produced the typical large metabolic disturbance whereas the intracapsular fracture—and this not in an elderly or feeble person—elicited a response no greater than that of trifling soft tissue trauma (Moore *et al.*, 1955).

Burns. A description of some of the changes occurring in burns occurs in Dr. [illegible] It will only [illegible] severe form of injury. Problems of water and electrolyte [illegible]bolism are complex owing to the development of considerable inflammatory oedema. There is a long period of negative nitrogen balance corresponding in duration to what is seen in long bone fractures rather than to other types of soft tissue injury. Here again in our experience evidence of increased adrenocortical activity disappears some time before the increased protein catabolism declines.

[1] *See pp. 167–81 above*

Nutritional Depletion and the Metabolic Response to Injury

Most forms of chronic disease lead to characteristic changes in body composition. Clinical examination reveals a loss of fat and muscular tissue and this is confirmed by more detailed measurements of body composition. The proportion of water by weight in the body rises indicating a reduction of the relatively anhydrous fatty tissue. There is an increase in body sodium relative to body weight and there is often an increase in the absolute measurements indicating a replacement of fat by extracellular fluid (McCance and Widdowson, 1951). Exchangeable potassium is reduced consequent on loss of lean tissue, especially skeletal muscle.

These gross changes in body composition not unnaturally affect the response to an inflicted injury. It has been fully demonstrated that the patient in poor nutritional state shows a relatively inconspicuous response. The catabolism of nitrogen is less in the first stage and the loss of weight often not so steep but there may be a more pronounced tendency to salt and water retention.

The excretion of 17 hydroxycorticoids in the urine is less than in healthy subjects.

Response to a second injury. Some of the most interesting demonstrations of the effect of a change of the nutritional state on the metabolic response have been in studies of two-stage surgical operations. The first operation is carried out on a patient in good nutritional health, the second two to three weeks later when he has not fully recovered from the depletion caused by the previous injury. If the two operations are of strictly equal severity, for example a lumbodorsal sympathectomy on each side, the contrast is striking In one such patient studied recently the first operation produced the typical response with a rapid catabolism of protein and fat and the characteristic serum electrolyte changes, namely a fall in sodium and a rise in potassium concentrations. There was also a considerable increase in the output of 17 hydroxycorticoids in the urine. On the occasion of the second operation performed 19 days later the metabolic disturbance was slight. The changes in nitrogen

Major soft tissue injuries. In these cases if the patient before injury was in good health there is a profound metabolic response. The duration and extent of protein catabolism is increased and a considerable quantity of fat is burnt. A prolonged period of sodium retention is frequently noted. The urinary excretion of 17 hydroxycorticoids is increased over a longer period. It is to be noted that this type of reaction also occurs after relatively minor trauma if sepsis subsequently develops, for example appendicectomy complicated by the development of a pelvic abscess.

Bone fractures. Cuthbertson in his early studies (1932) showed that there was a profound nitrogen loss after fracture of long bones. This has been confirmed in later studies. In injuries of this type there is a prolonged phase of protein catabolism despite adequate intakes of nitrogen and calories. By contrast the 17 hydroxycorticoid excretion is similar to that seen in soft tissue injury and only lasts a few days. The excessive protein catabolism may continue for a month—far longer than there is any evidence of increased corticoid excretion. In one series of cases an interesting contrast has been made between midshaft long bone fracture on the one hand and intracapsular fracture of the neck of the femur on the other. The former produced the typical large metabolic disturbance whereas the intracapsular fracture—and this not in an elderly or feeble person—elicited a response no greater than that of trifling soft tissue trauma (Moore *et al.*, 1955).

Burns. A description of some of the changes occurring in burns occurs in Dr. J. P. Bull's lecture printed earlier in this volume.[1] It will only be noted here that this constitutes an extremely severe form of injury. Problems of water and electrolyte metabolism are complex owing to the development of considerable inflammatory oedema. There is a long period of negative nitrogen balance corresponding in duration to what is seen in long bone fractures rather than to other types of soft tissue injury. Here again in our experience evidence of increased adrenocortical activity disappears some time before the increased protein catabolism declines.

[1] See pp. 167–81 above.

sodium and chloride in the plasma. The mechanism of this reduction in electrolyte concentration is also as yet undetermined. The fall is not due to any loss of these ions from the body. Dilution of the body electrolytes by the water retention may clearly be an important factor but the fall cannot be entirely eliminated by fluid restriction. Other possible explanations are a movement of water from the cells or alimentary canal into the extracellular fluid or a movement of sodium and chloride in the opposite direction. More detailed study of these points is still required.

The description and discussion have been almost entirely confined to the metabolic disturbances seen after injury in healthy adults. No attempt has been made to describe the variations in the metabolic response to injury seen in those with various types of disease. For instance, after cardiac operations gross disturbances in water and electrolyte metabolism may be seen (Wilson *et al.*, 1954b). The importance of understanding in the first place the sequence of events that occur in the healthy adult as a result of trauma needs no elaboration. An appreciation of what we may term the physiology of convalescence must form the scientific basis for the study of the abnormal response and for the intelligent care of the postoperative patient.

REFERENCES

BAUER, G. C H (1954) Metabolism of bone sodium in rats investigated with Na^{22} *Acta physiol scand.* 31, 334

BENEDICT, F G. (1915). A study of prolonged fasting. Carnegie Institution of Washington, Publication No 203

BENNET, E V. and MOORE, F. D (1951). The effects of surgical trauma and exogenous hormone therapy on the urinary excretion of 17-ketosteroids. *Surgical Forum, Amer Coll of Surg*, 1951. W. B Saunders, Philadelphia

BROWNE, J. S L (1950 Protein metabolism in acute and chronic disease and the relation of protein metabolism *to the excretion of glucocorticoids* *Proceedings of the first clinical A C.T.H conference*, p. 127. Ed. by J R. Mote. Churchill, London

excretion were less conspicuous and no change in serum electrolytes and output of 17 hydroxycorticoids was seen. There was, however, more marked sodium retention after the second operation (Moore *et al.*, 1955, case 8).

General Discussion

The changes after injury have been widely studied during recent years, particularly with regard to the chief body constituents such as protein, fat, sodium, potassium and water and several fairly definite patterns of response have been established. At the present time, however, relatively little is known regarding the mechanisms, control and significance of these metabolic changes.

The rapid catabolism of protein and fat presumably has as its primary purpose the provision of raw materials and energy for the healing of the wound. Browne (1950) introduced the concept that a 'loosening' of body nitrogen occurred to make it available for wound healing. It is significant that the healthy person who heals his wounds well after a single trauma and thrives clinically shows this catabolism most vigorously and the depleted patient who is more apt to fail in healing does not show a conspicuous catabolic reaction. The mechanism of control of this catabolic phase is obscure. A supply of adrenal cortical hormone is essential for its development but the meta- [illegible] solely to a hypersecretion of [illegible] n the bone injuries the period [illegible] r exceeded the duration of increased corticoid excretion and appeared to be related rather to the time required for new tissue formation. Indeed, in those types of injury in which there is a long period of repair, such as fractures and burns, it is tempting to postulate that the wound itself functions almost as an endocrine organ and releases some substance that sustains the catabolic reaction.

The interpretation of the changes in water and electrolyte metabolism seen after injury presents many problems. It seems probable that the conspicuous water retention may be related to the release of antidiuretic hormone. If this concept is true the hormone is being liberated in spite of a falling concentration of

MOORE, F. D. and BALL, M (1952) *The Metabolic Response to Surgery.* Thomas, Springfields, Illinois

MOORE, F. D, HALEY, H B, BERING, E. A, BROOKS, L and EDELMAN, I. S. (1952) Further observations on total body water. II. Changes of body

M

M , , ... BROOKS, L. and WILSON, G M (1954). Body sodium and potassium. III. Interrelated trends in alimentary, renal and cardiovascular disease *Metabolism*, 3, 334.

MOORE, F D, STEENBURG, R. W., BALL, M. R, WILSON, G. M and

Surg. 141, 145.

NORYMBERSKI, J. K, STUBBS, R D. and WEST, H. F. (1953). Assessment of adrenocortical activity by assay of 17 ketogenic steroids in urine *Lancet*, i, 1276

RATHBUN, E N. and PACE, N (1945) Studies on body composition. I. The determination of total body fat by means of the body specific gravity. *J biol. Chem* 158, 667

SCHLOERB, P. R., FRIIS-HANSEN, B J, EDELMAN, I. S, SOLOMAN, A. K. and MOORE, F. D (1950) The measurement of total body water in the human subject by deuterium oxide dilution, with a consideration of the dynamics of deuterium distribution. *J clin Invest.* 29, 1296.

VENNING, E H, HOFFMAN, M M and BROWNE, J. S. L. (1944) The extraction of cortin-like substances from human post-operative urine. *Endocrinology*, 35, 49

WILSON, G M, OLNEY, J M, BROOKS, L, MYRDEN, J A., BALL, M. and MOORE, F D (1954a) Body sodium and potassium II. A comparison of metabolic balance and isotope dilution methods of study. *Metabolism*, 3, 324

WILSON, G M, EDELMAN, I. S, BROOKS, L., MYRDEN, J. A, HARKEN, D E and MOORE, F D. (1954b). Metabolic changes associated with mitral

W

BURCH, G. E., RAY, C. T. and THREEFOOT, S A. (1953). Some theoretic considerations of electrolyte space measured by the tracer method in intact man. *J. Lab. clin. Med.* 42, 34.

CORSA, L., OLNEY, J. M., STEENBURG, R. W., BALL, M R. and MOORE, F. D. (1950). The measurement of exchangeable potassium in man by isotope dilution. *J. clin Invest.* 29, 1280.

CUTHBERTSON, D P. (1932). Observations on the disturbance of metabolism produced by injury to the limbs. *Quart J. Med.* (N.S.) 1, 233.

DAVIES, R. E., KORNBERG, H. L. and WILSON, G. M. (1952). Non-exchangeable sodium in the body. *Biochim biophys. Acta*, 9, 703.

EDELMAN, I. S., OLNEY, J. M., JAMES, A. H., BROOKS, L. and MOORE, F. D. (1952a). Body composition: studies in the human being by the dilution principle. *Science*, 115, 447.

EDELMAN, [illegible] S., HALEY, H. B, SCHLOERB, P R., SHELDON, D. B, FRIIS-HANSEN, B. J., STOLL, G. and MOORE, F. D. (1952b). Further observations on total body water. 1. Normal values throughout the life span. *Surg. Gynec. Obstet.* 95, 1.

EDELMAN, I. S, BROOKS, L. and MOORE, F. D. (1952c). Endogenous fat metabolism studied by heavy water dilution and nitrogen balance. *J. clin Invest.* 31, 626

EDELMAN, I S, JAMES, A H., BADEN, H and MOORE, F. D. (1954). Electrolyte composition of bone and the penetration of radiosodium and deuterium oxide into dog and human bone. *J. clin. Invest* 33, 122.

FRANKSSON, C, GEMZELL, C. A. and VON EULER, U. S (1954). Cortical and medullary adrenal activity in surgical and allied conditions *J. clin. Endocrinol.* 14, 608.

HASTINGS, A. B. (1941). The electrolytes of tissues and body fluids. *Harvey Lecture*, 36, 91.

HOWARD, J. E (1945) Protein metabolism during convalescence after trauma *Arch. Surg* 50, 166

INGLE, D. J. (1953). The role of the adrenal cortex in homeostasis *The Suprarenal Cortex*, ed. J M Yoffey, p 177. Butterworth, London.

245.
ing
id,
cell mass, and fat, and some of its applications to physiology and medicine *Proc. roy. Soc*, B, 138, 115.

MILLER, H, MUNRO, D S, RENSCHLER, H E. and WILSON, G M. *Obser-*
... of exchangeable sodium in
and physi-
ions, London.

grouped under two headings: (1) radiation dysplasia, and (2) radiation neoplasia.

It must be emphasized that it is unwise to attribute minor changes in the radiographic and microscopic picture seen in a bone to radiation unless control material is available. In the case of gross radiography, pictures of bones from individuals of the same age who have lived under similar conditions should be available for comparison. In the case of histology or micro-radiography sections taken from the normal bones at the same level in individuals of the same age or in the case of animals of the same age and species should be used as controls. The variations in different bones depending on the age of the animal or man and the level at which the section is taken are remarkable (Amprino and Bairati, 1936; Owen, Jowsey and Vaughan, 1955).

External Radiation

1. *Radiation dysplasia*

The term radiation osteitis was first used by Ewing to describe

. .

. .

not necessarily any infective element present it is more logical to describe the bone changes as dysplasia. The lesions seen in bone vary according to the age of the bone at the time of irradiation, the magnitude of the dose of radiation and the presence or absence of associated but independent sepsis. In young people when growth is active there is retardation of growth; in young and old subjects the dysplasia may be complicated by fracture and osteomyelitis.

Retardation of growth. Retardation of growth may be severe (Bisgard and Hunt, 1936, Murphy and Berens, 1952). Frantz (1950) described a boy of 16 who had a shortening of $9\frac{3}{4}$ inches in one femur. At the age of $2\frac{1}{2}$ weeks he received approximately 3,000 roentgens given over a short period for treatment of a haemangioma over the distal femoral epiphysis. There was also a decrease in shaft diameter and cortical thickness Neuhauser and his colleagues (1952) have recently reviewed a group of

XII

Radiation Injury to Bone

JANET VAUGHAN

Introduction

It has long been known that severe, sometimes fatal, damage to bone tissue may be produced by both external and internal radiation. The possibility of such damage has become increasingly important with the increased use of radiation therapy and of radioactive isotopes as therapeutic, research, or industrial tools.

The effects of irradiation on bone have been studied in both experimental animals and in human beings. Though much can be learnt from experimental work on animals, the results are not strictly comparable with clinical findings in man, since (*i*) there are considerable species variations; (*ii*) much of the work has been done on small animals and the method of bone growth in rats and mice is unlike that in man; (*iii*) a much longer latent period is possible in man than in animals. Experimental results in animals must therefore be used with reservation in interpreting clinical findings in man.

It is proposed in the following pages to summarize what is known of the clinical picture of radiation damage to bone depending as far as possible upon experience in man, and then to discuss the reasons that make bone tissue so liable to radiation injury.

I. Clinical Picture of Radiation Injury

The pathological changes produced in bone by external and internal radiations do not appear to differ, though for convenience they are discussed separately. These changes can be

a comparable age group in non-irradiated women There is no reason to believe secondary deposits are involved since in many cases careful biopsy and autopsy results have revealed no growth and typical radiation dysplasia (Baensch, 1927; Dalby

of 40 and 50, though the average age is about 63 (Kok, 1953). The youngest is 34 (Stampfli and Kerr, 1947). Fracture following prophylactic irradiation after operations for carcinoma of the breast has also been recorded in the ribs and clavicle (Peck, 1939, Slaughter, 1942; Smithers and Rhys-Lewis, 1945), and in the pelvic bones (Peck, 1939; Gratzek *et al.*, 1945), but is rare.

Osteomyelitis The relationship between infection of bones and radiation damage is so close that in early experiments where an attempt was made to induce neoplasms by radiation it was common practice to infect the bone simultaneously with the tubercle bacillus (Lacassagne and Vinzent, 1929). While it is now recognized that radiation alone will cause bone dysplasia and neoplasia it is also clear that bone devitalized by radiation is peculiarly susceptible to infection (Ewing, 1926). The close relationship of necrosis to osteomyelitis so often seen in the jaw bones of patients irradiated for oral malignancy was probably first noted by Regaud (1922). Daland (1949) goes so far as to say that teeth and irradiation therapy are incompatible. Whenever deep X-ray therapy is to be directed to lesions of the mouth he considers that the teeth should first be removed since they are a source of sepsis. In a series of 1,819 patients with intra-oral cancer treated with radiation, 235 developed osteo-radio necrosis. If the lip cases were excluded the figure increased to 20 per cent (Daland, 1949). Recession of the mucosa occurs around over-irradiated teeth, the blood supply is impaired, pyorrhoea and sepsis follow which then spreads to bone tissue already devitalized by irradiation. Daland quotes the case of a man in whom necrosis of the jaw developed seven years after successful irradiation of his tongue for carcinoma. Secondaries

34 children 2–3 years after irradiation over the spine for retroperitoneal or paravertebral neoplasm or benign haemangioma. Exposure to more than 2,000 roentgens invariably produced disturbance of growth irrespective of the age of the child when treated. The roentgenological findings described by different authors may be summarized (Neuhauser *et al.*, 1952; Whitehouse and Lampe, 1953).

(*i*) horizontal transverse lines of increased density parallel to the epiphyseal plates, like the 'lead line' of lead poisoning;

(*ii*) gross irregularity of the vertebral epiphyseal cartilage plates;

(*iii*) scoliosis or lateral curvature;

(*iv*) excess of benign exostosis cartilaginae;

(*v*) uneven development of bone.

Fracture. Fracture following external irradiation occurs most commonly in the femoral neck in women who have received radiation treatment for malignant conditions of the pelvic organs. There appear to be only three cases recorded in men (Bonfiglio, 1953). The characteristic features of post-irradiation femoral fractures are:

(*i*) usually there is antecedent pain without radiographic evidence of the fracture which occurs later unassociated with trauma;

(*ii*) there may be no chronic pain but sudden acute pain without trauma, and a fracture is seen on the radiograph;

(*iii*) the interval between completion of radiation and the fracture may be considerable;

(*iv*) if a radiograph is taken before fracture occurs, an irregular area of bone condensation in the femoral neck is often seen.

The recorded interval elapsing between radiation and fracture varies from 4 months to 6½ years. There are several instances of bilateral fracture though not necessarily occurring at the same time (Baensch, 1927; Kalayjian, 1938; Strauss and McGoldrich, 1941; Barden, 1943; Gratzek, Holmstrom and Rigler, 1945, McCrorie, 1950) The left femur is said to be fractured more often than the right (Kok, 1953). The incidence of radiation fractures in the femur in the series of cases recorded varies from 0·1 per cent to 3·2 per cent which is higher than in

a comparable age group in non-irradiated women. There is no reason to believe secondary deposits are involved since in many cases careful biopsy and autopsy results have revealed no [illegible]

of 40 and 50, though the average age is about 63 (Kok, 1953). The youngest is 34 (Stampfli and Kerr, 1947). Fracture following prophylactic irradiation after operations for carcinoma of the breast has also been recorded in the ribs and clavicle (Peck, 1939; Slaughter, 1942; Smithers and Rhys-Lewis, 1945), and in the pelvic bones (Peck, 1939; Gratzek *et al.*, 1945), but is rare.

Osteomyelitis. The relationship between infection of bones and radiation damage is so close that in early experiments where an attempt was made to induce neoplasms by radiation it was common practice to infect the bone simultaneously with the tubercle bacillus (Lacassagne and Vinzent, 1929) While it is now recognized that radiation alone will cause bone dysplasia and neoplasia it is also clear that bone devitalized by radiation is peculiarly susceptible to infection (Ewing, 1926). The close relationship of necrosis to osteomyelitis so often seen in the jaw bones of patients irradiated for oral malignancy was probably first noted by Regaud (1922). Daland (1949) goes so far as to say that teeth and irradiation therapy are incompatible. Whenever deep X-ray therapy is to be directed to lesions of the mouth he considers that the teeth should first be removed since they are a source of sepsis In a series of 1,819 patients with intra-oral cancer treated with radiation, 235 developed osteo-radio necrosis. If the lip cases were excluded the figure increased to 20 per cent (Daland, 1949) Recession of the mucosa occurs around over-irradiated teeth, the blood supply is impaired, pyorrhoea and sepsis follow which then spreads to bone tissue already devitalized by irradiation. Daland quotes the case of a man in whom necrosis of the jaw developed seven years after successful irradiation of his tongue for carcinoma. Secondaries

were not found and he attributes the necrosis to infection spreading from abscessed teeth which should have been removed before treatment.

2. Radiation neoplasia

The relationship of radiation dysplasia and neoplasia is still obscure. The former may certainly occur without the latter but it is probable that neoplastic change is always preceded by dysplasia.

Thirty-nine instances of sarcoma arising in bone following external irradiation have been traced. There are undoubtedly others in which the aetiological effect of radiation has been obscured by the long latent period between exposure and tumour development. The first 16 cases followed radiotherapy of tuberculous arthritis. The latent period between ending treatment and development of tumour was 3–11 years. Cahan and his colleagues have described 11 cases from the experience [illegible] microscopic [illegible] ature of the [illegible] concerning the tumour. The latent period varied between 5–22 years with an average of 11·2 years. Other isolated cases have been described. Excluding one case where treatment was given over a long period, the latent period varied from 3–22 years with a mean of 8·6 years. The ages at which a tumour developed varied from 9–62 years, the majority occurring between 15 and 25. Jones (1953) claims that bone sarcoma in the population as a whole has a wider age spread with a double age peak, one between 15 and 25 and one between 45 and 55. Increasing pain and swelling in the region of a bone previously irradiated should arouse suspicion, though radiologically the lesion may not be altogether typical of bone sarcoma. Secondary metastases to the lung have been found in many of the recorded cases (Beck, 1922; Jaruslawsky, 1929; Hellner, 1937; Cahan *et al.*, 1948).

Radiation dosage. It is extremely difficult to calculate the radiation dosage received by bone tissue in the early cases that developed tumours after skeletal irradiation. In Cahan's (Cahan *et al.*, 1948) more recent series, excluding two doubtful

cases, the lowest dose recorded was 3,000 roentgens but the majority received a higher dose.

INTERNAL RADIATION

The clinical picture of injury due to internal radiation is based on a study of patients who have ingested radium, mesothorium or a mixture of the two, either as the result of their occupation (Hoffman, 1925; Martland *et al.*, 1925; Martland, 1929; Aub *et al.*, 1952) or for therapeutic purposes (Looney, 1951; Aub *et al.*, 1952). Experience with experimental animals suggests that other radioactive elements would give the same clinical picture (Lisco, Finkel and Brues, 1947; Brues, 1949; Finkel, 1953).

The first deaths that occurred in the radium dial painters of the 1914 War 4–6 years after leaving work were due to profound anaemia, sometimes associated with severe necrosis of the jaw. In later years the dial painters developed bone lesions particularly sarcoma (Martland, 1929; Aub *et al.*, 1952). Aub and his colleagues (1952) have recently reviewed 26 patients who sought medical advice because of bone pain or fracture and were known to have ingested radium or radium and mesothorium Some had tumours, some fractures, some osteomyelitis, and others varying degrees of radiation dysplasia demonstrable on X-ray examination.

1. *Radiation dysplasia*

It is claimed that the chronic effects of internal radiation on bone tissue may in their early stages be symptomless and only found on routine radiological examination of the skeleton (Schwartz, Makepiece and Dean, 1933); on the other hand dysplasia without fracture may give rise to a severe and crippling bone pain requiring heavy sedation (Looney, 1951, Aub *et al.*, 1952). Though scepticism has been expressed as to the significance of certain of the minor changes described (Vaughan, in the press) it is clear that unexplained bone abnormalities seen on X-ray examination should always raise the possibility of radiation injury. Looney (Looney, 1951; Looney and Woodruff, 1953) has described many of the minor radiological changes in

the skeleton seen on radiographic examination which he considers characteristic of radiation damage. His patients were inmates of an asylum who were examined because they had been known to have received injections of radium in solution for therapeutic purposes many years before. Unfortunately no control pictures on other asylum inmates known to have been bedridden and to have taken the same sort of diet over a long period of time are available for comparison. The lesions described on radiological examination are (Looney, 1951; Aub *et al.*, 1952).

(*i*) coarsening of bone trabeculae;

(*ii*) areas of increased and decreased density;

(*iii*) longitudinal areas of decreased density in the long bones;

(*iv*) varying degrees of distortion or destruction of normal bone pattern;

(*v*) damage to the ends of the long bones in young people whose epiphyses have not closed (Frantz, 1950);

(*vi*) destruction and collapse of either vertebrae or long bones subject to weight bearing (Aub *et al.*, 1952).

(*vii*) small well-defined areas of decreased density in the skull.

Such skull lesions have led in the past to the diagnosis of multiple myeloma especially when they are found in a patient associated with severe anaemia and possibly abnormal blood cells and Bence Jones protein in the urine. Death may finally occur in such patients from the blood dyscrasia which develops in the terminal stage, bone pain and skeletal abnormalities having been present for many years.

Fracture. Single or multiple fractures may occur. These usually heal but slowly and there is much callus formation. The callus may be radioactive showing that the radium is mobilized together with calcium in the formation of new bone—adjacent bone may be non-radioactive. All stages of dysplasia give rise to crippling bone pain which is often diagnosed either as rheumatism or neurosis. After long periods Aub (Aub *et al.*, 1952) states the dysplasia may heal. It remains to be seen, however, whether tumours do not subsequently develop in these patients.

Osteomyelitis. In his early cases of radium poisoning Martland described several instances of severe necrosis and infection of the

jaw (Martland *et al.*, 1925). Osteomyelitis secondary to radiation dysplasia has also been described in the temporal bone (Aub *et al.*, 1952). As in the case of osteomyelitis secondary to external radiation, infection spreads rapidly from an already septic tooth to necrotic bone. Once the teeth are removed the jaw usually though not invariably heals. Changes in the teeth unassociated with sepsis are described by Looney (Looney, 1951) as so characteristic as to lead a dentist to suggest the possibility of radium poisoning. On radiographic examination the teeth have a honeycombed appearance and to the naked eye look pinkish owing to decrease in the density of dentine between the red pulp and the outer enamel covering. Those who have studied many cases of radium ingestion speak of the teeth as 'pink teeth'.

2. *Radiation neoplasia*

The outstanding characteristic of bone tumours due to internal irradiation in man is the long latent period between ingestion of the radioactive element and the development of the bone lesion (Brues, 1949). Physiological reasons that may in part account for this are discussed elsewhere (page 218).

In man tumours in the skeleton are hitherto only known to have resulted from the ingestion of radium or a mixture of radium and mesothorium, presumably because exposure to other bone-seeking isotopes has only recently become possible. With the increasing exposure to radioactive isotopes that is now occurring it is only to be expected that skeletal tumours due to the presence of other bone-seeking isotopes will occur. Brues has described radioactive strontium as 'par excellence a producer of bone tumours' (Brues, 1949). In mice the relative efficiency of beta-emitting isotopes such as strontium in producing tumours compared with alpha-emitting isotopes like radium is 1 : 10 on a millicurie basis. The maximum permissible body burden of radium is 0·1 μc., the maximum permissible body burden of strontium is 1·0 μc.

Tumours in or near bone in mice following exposure to ^{89}Sr, a beta emitter, were predominantly osteogenic sarcoma—a few tumours of mesenchymal or endothelial origin occurred.

Multiple primary tumours were found more frequently with increasing dosage. Tumours were found in all parts of the skeleton though following radioactive strontium, yttrium or cerium they were said to be more common in the long bones than in the spine (Lisco *et al.*, 1947). Multiple tumours following injection of plutonium, an alpha emitter, were the rule when the dose was large and are likely to be found still more frequently when microscopic examination is carried out.

It has been claimed that in mice (Lisco, Finkel and Brues, 1947) the incidence of bone tumours is approximately proportional to the dose of ^{89}Sr administered, the latent period being not less than 200 days. Of mice that received 5 μc. of ^{89}Sr per gram body weight and survived 200 days, 14 per cent developed bone tumours. Brues concludes that it is impossible to produce bone tumours with small doses in short-lived animals. The low dose of radium that will give rise to radiation neoplasia in man is consistent with the long period available in which to do so. He suggests that each quantity of absorbed radiation confers on the tissue absorbing it a probability of tumour formation which is without a limit in time once the latent period is passed. The latent period increases as the dose decreases. Knowing this, the high tumour incidence in animals following relatively high doses of all the bone-seeking isotopes is a ghastly warning of what we may expect in either the survivors of Hiroshima or any other atomic disaster quite apart from individual accidents in industry and laboratory. Excluding tumours of the jaw which may have been complicated by a previous osteomyelitis the latent period before development of symptoms and ingestion in Aub's group of 8 cases that have already developed tumours following ingestion of radium or radium and mesothorium varied from 12–26 years. In spite of what has been said about experience in mice the length of the latent period in man is not entirely dependent on the level of the body burden. For instance, in Aub's series the patient with the highest skeletal content had a latent period of 25 years
[illegible] had appeared
[illegible] to
[illegible] cise

statement as to the maximum burden of radium or other isotope the body will tolerate without the risk of tumour production. The clinical picture on which such an assessment might be based is complicated by the presence in so many cases of mesothorium as well as radium (Aub *et al.*, 1952). One patient with a body burden as low as 0·8 μg. at the time of death developed a tumour after a latent period of 25 years. The recommended permitted body burden of radium is 0·1 μg. This is clearly not conservative if theoretical considerations are also taken into account (Spiers, 1953). Indeed, on a theoretical basis a figure of 0·02 μg. might be safer (Spiers, 1953).

The majority of tumours following injection or ingestion of radium are described as osteogenic sarcoma, though it is often [illegible]

by histology, and one of periosteal fibrosarcoma arising in the knee joint. The following bones have been involved the femur and pelvis most frequently, but also the scapula, ribs, humerus, jaws, metacarpals and orbit. Martland (1931) recorded one instance when there were two primary tumours in the same patient, one in the orbit and the other in the pelvis Generalized metastases, especially to the lungs, are common.

Pathological changes in radiation dysplasia and neoplasia. The changes in bone resulting from radiation are little understood, though there are many descriptions of both the gross and the histological picture (Gates, 1943; Heller, 1948). The picture described, however, is usually the terminal stage following heavy local radiation and the exact means, in terms of bone pathology or distorted physiology, by which this picture has been reached remains obscure. There is at present no evidence that there is any fundamental difference between the effect of external and internal radiation on bone structure as seen on histological examination. There are, however, as might be expected, differences between the end effects on adult cortical bone and the effects on the still actively growing epiphyses. Death of all bone cells occurs if radiation is heavy. Chondroblasts are said to be the most sensitive cells and osteoclasts the

least. The capacity, however, of even heavily irradiated bone to repair, though bone formation is abnormal, is remarkable (Heller, 1948).

Active epiphyseal bone. In animal experiments the earliest changes seen in irradiated bone occur in the swollen cartilage cells which first become abnormally swollen and irregular in their arrangement (Heller, 1948; Kligerman, 1950). Later normal interdigitation between cartilage and metaphyseal spongy bone may lead to apparent separation (Heller, 1948) followed by renewed and often irregular growth. The result of the irregular growth is some increase in the length of the bone, new bone being formed above that injured by radiation which is apparently displaced. The band of affected cartilage cells appears to show increased calcification. Heller describes abnormal amounts of calcified cartilage embedded in atypical acellular and fibrous bone in the 'lead line' seen in roentgenograms. In animals this lead line may appear on longitudinal sections of the bone as a bridge of bone across the narrow cavity suggesting failure of resorption of metaphyseal trabeculae in the area of radiation damage though resorption has occurred in a normal manner above. Failure of resorption in the normal process of remodelling would also account for the persistence of excess of calcified cartilage (Owen, Jowsey and Vaughan, 1955) and the so-called 'lead line' seen in the radiograms of bone of both animals and children. Experiments using radioactive strontium have demonstrated the persistence of the ^{90}Sr in high concentration in unresorbed calcified cartilage at distances from the epiphyseal plate dependent on the time of death in relation to administration (Plate X, Figure 1).[1]

Adult bone. Ewing has described the following gross changes in cortical bone following external radiation. 'There is marked thickening of the corticalis and the new bone is not laid down in lamellae but projects in broad irregular elevations into the marrow cavity which is also partly obliterated by increase in fibrous tissue and bone trabeculae. There is also thickening of the periosteum, sometimes to as much as 9 inches.' (Ewing, 1926.) At the histological level there is increased formation of

[1] The plates referred to in this lecture will be found between pages 208–9

fibrous tissue, loss of metachromatic staining associated with hyalization of collagen and gross disturbance of the normal bone pattern with fibrosis of the vessels. Whether the histological picture is due to interference with the blood supply or to bone cells is disputed. On balance the latter seems more probable (Ross, 1936; Dunlap *et al.*, 1944; Heller, 1948; Spiers, 1949; Fink, [illegible]; Aub *et al.*, [illegible]; Bonfiglio, [illegible]; Kolr, [illegible]; Spiers [illegible]

I [illegible]

c [illegible]

and vessels is probably relevant to the pathological picture (Rutishauser and Majno, 1951; Wassermann, 1952; Jackson, 1953).

Special interest attaches to the bone changes observed in patients who have ingested radium or radium and mesothorium and died many years later. Clearly any interpretation of a pathological process that may have been going on for many years is difficult. Both Looney and Woodruff (1953) and Hoecker and Roofe (1949, 1951) and Aub and his colleagues (1952) describe areas of increased bone density and areas of bone disorganization as evidenced either by absence of evenly distributed lacunae or irregular atypical spaces (Looney, 1951) or simply extreme decalcification of necrotic bone resulting in fracture. Autoradiographic studies of bone sections show that radium is laid down in scattered areas of heavy concentration. Hoecker and Roofe (1949, 1951) found that the deposition of radium was associated with the denser sections of the bone and with one particular Haversian system, while in other and adjacent sections there was a negligible amount present. The maximum density of radium as indicated by the number of alpha tracks per unit area appeared to vary by a factor of only about 1·5 from one type of bone to another. For instance in bones removed from one patient, concentration in the periosteal portion of the os pubis were about 1 5 times as great as in the periosteal part of the femur. On the other hand the frequency of localization in different bones may vary by a factor of nearly 25. In the corticalis of the femur only about 2 per cent of the Haversian canals contained radium as compared with 50 per cent in the os pubis. In another patient whose total body

burden at autopsy was estimated to be 50 µg. the concentration in the Haversian systems in the shaft appeared to be about six times as dense as those in comparable bone from a patient estimated to contain 8 µg. at autopsy. Looney and Woodruff (1953) emphasize that though the radium occurs in scattered concentrations it is more unevenly distributed than Hoecker and Roofe suggest. They found irregular rather than regular distribution round any Haversian system. They also found patchy concentrations in trabecular bone and in cement lines—areas of bone destruction were sometimes associated with deposition of radium and sometimes occurred in non-radioactive bone. They also describe macroscopic areas of destruction usually unassociated with radium deposition. Hoecker and Roofe (1951) also describe considerable abnormal resorption and indications of calcium deficiency associated usually with rickets.

In the region of a developing sarcoma Hoecker and Roofe (1951) emphasize that though calcium was still present, minimum amounts of radium were found. The significance of the manner of distribution of radium is discussed on page 216.

II. Radiation Dosage

Before discussing the peculiarities of bone that affect its susceptibility to radiation a simple definition of certain terms used in discussion of radiation dosage must be given.

1. *Character of radiations*

The radiations involved may be alpha, beta, gamma, or X-rays or any combination of these. These are all what are called ionizing radiations, i.e. when they pass through matter whatever their source, their principal means of losing energy is by the ejection of electrons from the atoms of which the matter is composed The atom is left positively ionized and as a result *there is chemical change in the molecules of* which the atom is a part. The 'specific ionization' thus produced is the number of ionizations per micron of path-length of the radiation. Different types of rays and the rays of different isotopes vary in the specific ionization they cause. Alpha rays produce high specific ionization along their path and are therefore the most dangerous

PLATE X

F … graph left on cross-section shaft tibia rabbit aged 5-7 weeks when injected ^{90}Sr (1000 μc /kg), killed 6 months later Note heavy reaction in metaphyseal bone in anterior wall in remnants of calcified cartilage and adjacent bone (The amount of cartilage present is due to failure of resorption dependent on radiation damage) (× 60)

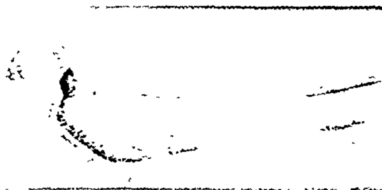


FIG 2 Autoradiograph longitudinal section tibia of rabbit aged 3-4 months injected ^{91}Y (80 μc kg), killed 10 minutes after injection Note irregular concentrated reaction (i) in region of epiphyseal plate (ii) throughout cortical bone (× 3)

PLATE XI



FIG. 3. Autoradiograph left on cross-section cortical bone rabbit aged 5–7 weeks, injected ^{91}Y (1000 μc/kg). Note (i) heavy reaction round small vessel, (ii) no reaction round building sites or over rest of bone. (× 260)



FIG 4 Autoradiograph left on cross-section cortical bone rabbit aged 6 months, injected ^{91}Y (1000 μc /kg), killed 21 days Note (i) heavy reaction over cement line, (ii) heavy reaction round edge of Haversian canal. (× 260)

Plate XIII



Fig 5. Autoradiograph from longitudinal section lower end of femur rabbit aged 5–7 weeks, injected ^{90}Sr (80 μc /kg), killed 10 minutes later. (× 2)

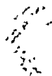




Fig 6 Autoradiograph from longitudinal section lower femur end of rabbit aged 5–7 weeks, injected ^{90}Sr (80 μc /kg), killed 18 months later. (2)

Plate XIV

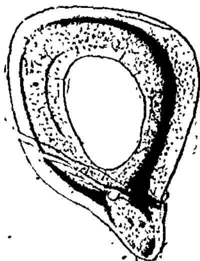


Fig 7 Autoradiograph left on cross-section middle diaphysis tibia rabbit aged 5–7 weeks when injected ^{90}Sr (1000 μc./kg), killed 6 months later Note persistence of heavy reaction in endosteal and periosteal bone not resorbed in process of remodelling (× 10)



periosteal bone (× 60)

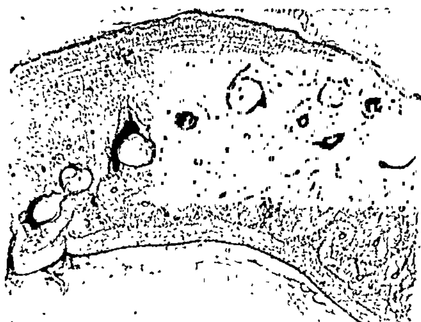


FIG. 9 *Autoradiograph left on section from which microradiograph prepared* Note heavy reaction in areas of low calcification ro building sites (× 60)



FIG. 10 Microautoradiograph cross-section cortical bone, monkey, 4 years, injected ^{90}Sr (1000 μc /kg), died 23 days Note (i) resorption cavity with high calcification, (ii) building sites with low calcification on one side (× 260)

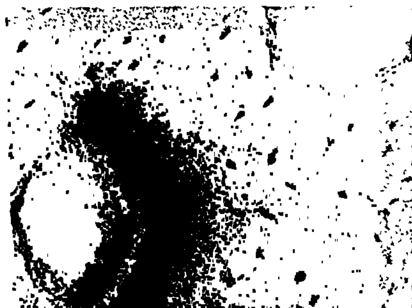


FIG. 11. Autoradiograph left on section from which microradiograph pre- i) heavy reaction in area of low calcification, (ii) no reaction in lcification (260)

PLATE XVII

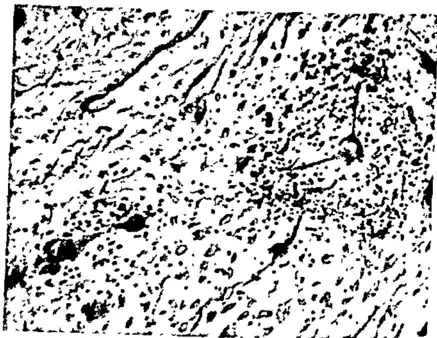


FIG. 12 Autoradiograph left on cross-section diaphysis tibia rabbit 3 months old, injected ^{35}S (3000 μc kg), killed 21 days later Note reaction due to ^{35}S at some distance from Haversian canal (× 140)

radiations in their effect on biological systems. Beta rays have a much lower specific ionization and are therefore less dangerous. X-rays and gamma rays are electromagnetic waves, gamma rays being of shorter wavelength, i.e. of higher energy and therefore more penetrating than X-rays. They both produce their effect in tissue by the production of high-speed electrons. It is generally agreed that radiation damage to tissues is dependent upon chemical changes produced by ionization (Lea, 1946; Gray, 1953).

2. *Radioactive decay*

The atoms of a radioactive substance disintegrate in such a manner that a certain definite proportion of the number of atoms initially present decays in a given time. The time required for half the atoms to decay to the daughter product is known as 'the half life' and is absolutely characteristic of that particular disintegration process.

Certain of these daughter products may be themselves radioactive and will then decay with different half lives until a stable non-radioactive isotope results. Values of the half life for different radioactive isotopes range from fractions of a second to many years and for obvious reasons those with a long half life are the more dangerous.

3. *Energy of radiation*

The energy emission associated with each disintegration process is definite and characteristic of that particular substance. For alpha particles this corresponds to the energy of the particle; for beta particles to the maximum energy of the particle, the actual energy varying from 300 up to this maximum with an average at about one-third of the maximum and for gamma rays to the wavelength of the electromagnetic radiation of photon energy This is expressed in 'MeV' (millions of electron volts) units.

4. *Relative biological efficiency*

The biological effectiveness of a tissue dose delivered by electrons (beta rays or secondary electrons in the case of X- or gamma rays) in practice does not vary much with specific

ionization, as the change in specific ionization in this range is less than 10 : 1 while the difference between alpha particles and beta particles is of the order of 1000 : 1. It is therefore at present assumed for protection purposes that all beta rays, X-rays and gamma rays have the same biological effectiveness. Since most of the directly applicable information on protection has been obtained from a study of the occupational exposure of radiologists to ordinary X-rays up to 250 kV this X-ray energy range is used as a point of reference—specific ionization within this range is nearly constant. Radiations in this range are assumed to have a biological effectiveness of 1. The biological effectiveness of other types of radiation which have different specific ionizations are related to X-rays. This is spoken of as the *r.b.e. or relative biological effectiveness.* For instance the relative biological effectiveness at near tolerance level in female mice for the alpha radiation from plutonium 239, polonium 211 and uranium 232 compared to radium is respectively 3, 2 and 1. Following a dose of 5·0 μc. of plutonium per kilogram body weight *85 per cent of the surviving animals had malignant bone* tumours compared with 30 per cent given a similar dose of radium.

5. *Units of dosage*

Amounts of radioactive material are expressed as curies. The *curie* is a unit of radioactivity defined as the quantity of any radioactive nucleotide in which the number of disintegrations per second is $3{\cdot}7 \times 10^{10}$.

Units of dose. Hitherto the commonest unit of quantity of *radiation used has been the roentgen—more recently others* have been introduced—the rad, the rep and the rem.

The roentgen was the unit adopted (Recommendations of the International Commission on Radiological Units, 1928 and 1937) for the measurement of quantity of X- or gamma radiation. *It is defined in terms of the ionization produced in air* under standard conditions and corresponds to an absorption of 83·7 ergs/gm. of air. When measuring doses in roentgens it must be appreciated that this is a measure of the amount of radiation reaching the point detected by its absorption in air.

Now tissue (and particularly of course bone) has neither the same atomic number nor electron density as air so that the energy absorption per unit mass of tissue for one roentgen of radiation will in general not be 83 7 ergs/gm., and will vary with the energy of the radiation and the nature of the particular tissue concerned. Thus for 1 roentgen of radiation the energy absorption in soft tissue (or water) varies from about 80 ergs/gm. at low voltage to about 93 ergs/gm. for radium gamma rays. For materials of higher atomic number such as bone the energy absorption per roentgen may be many times greater with low voltage radiation but the difference between bone and soft tissue will be small for radiation of 1–10 MeV.

In some cases where doses have been stated in roentgens this variation of energy absorption has not been taken into consideration.

The Rep. The roentgen is a unit which is only applicable to measurement of X- or gamma radiation. In order to provide a similar unit for the measurement of particle radiations the rep (roentgen—equivalent—physical) was suggested. This has been defined as the quantity of particle radiation giving an absorption in air equal to that of 1 r. of X-rays, i e. 83·7 ergs/gm. (Marinelli, Quinby and Hine, 1948). As the loss of energy from charged particles depends only on the mass of material traversed and not on its nature or atomic number the energy dissipation corresponding to 1 rep will be 83 7 ergs/gm. for all materials.

Confusion has sometimes been caused by workers using an absorption of 93 ergs/gm. to represent one rep, that is the energy absorption in soft tissue for 1 roentgen of radium gamma radiation However, this is not in agreement with the original definition but corresponds to the 'energy unit' suggested by Gray and others (1940).

The Rad. It has recently been agreed (Recommendations of the International Commission on Radiological Units, 1953) that dose should be measured in absolute energy units. A new unit 'the rad' has now been adopted as the unit of absorbed dose and corresponds to an absorption of 100 ergs/gm of the medium concerned.

All doses stated in rads will therefore correspond to the same energy absorption per unit mass whatever the radiation or medium concerned. However, the measurement of these doses must still, in most cases, depend on measurement of ionization in gas in a small cavity, and the calculation of energy absorption in terms of rads may be by no means easy or very accurate. Thus the adoption of the new unit does not necessarily imply that in future all doses will be measured and stated accurately.

The Rem. Even if we are able to measure dose in rads (or ergs/gm.) we shall not always expect to find the same biological result. Such physical features as the density of ionization along the particle track and such biological features as varying tissue sensitivity must affect the final response to the radiation exposure. The rem (roentgen—equiv.—man) attempts to allow for this variation and will be given by the tissue dose in rads multiplied by the relative biological efficiency for the particular radiation and biological effect concerned.

The maximum permissible dose of isotopes behaving like radium is calculated in relation to the maximum permissible body burden of radium, namely 0·1 μc. (International Committee on Radiological Protection, 1951). In other cases the m.p.d. is based on a calculation of that burden in the body which will result in an average biological dose of ionizing radiation equivalent to 0·3 rad per unit to the most sensitive organ in which concentration of the radioactive isotope occurs. Radium is at present used as the standard because it is the only radioactive isotope of which there is knowledge of its clinical effects in man

In practice assessment of radiation dose may become extremely complicated. For instance in the case of the radium dial painters many of them ingested a mixture of radium and mesothorium, but the proportions in the mixture were variable (Aub *et al.*, 1952). Radium has an extremely long half life (1,600 years), emits alpha particles and has a large range of radioactive decay products. Mesothorium has a short half life (6 7 years) and also a range of decay products which emit alpha, beta and gamma rays. The metabolic pathway within the bone of the decay products of mesothorium probably differs

from that of mesothorium itself (Aub *et al.*, 1952). In other words the possible injury received by bone tissue exposed to radium and mesothorium is extraordinarily complex.

III. Characteristics of Bone that influence its Susceptibility to Radiation Damage

Osseous tissue has certain peculiarities that make it particularly susceptible to radiation injury and which affect considerations of dosimetry. These may be summarized as (1) the non-homogeneous character of bone; (2) the affinity of osseous tissue or tissues closely associated with bone for a wide range of elements; (3) the methods of bone growth; (4) presence of extensive crystal surface

1. *The non-homogeneous character of bone*

The roentgen, the unit hitherto most commonly used to express radiation dose, is based on the ionization produced in air by the passage of X-rays (Spiers, 1946). In considering the dose received by a tissue element, it has often been assumed that the body is made up of homogeneous material, usually water or a water equivalent. Bone, however, consists of a highly mineralized protein matrix in which are embedded widely separated living cells in cavities of variable size. The absorption of X-rays or gamma radiations depends on the effective atomic number of the absorbing material. The highly mineralized protein matrix absorbs far more energy than soft tissues (Spiers, 1949) The 'ionization' occurring therefore in soft tissues at an interface between it and mineralized bone or inside small cavities within the bone is very much higher than might be expected from the dose originally expressed in roentgens (Spiers, 1950; Munson, 1950). The smaller the cavity the greater increase in average ionization per unit volume within the cavity (Spiers, 1949). In attempting to estimate the dose of radiation received by bone tissue from any source, whether internal or external, two factors in bone itself must therefore be remembered—(i) the effective atomic number of the inorganic bone, and (ii) the size and location of the soft tissues within it. The 'permissible' dose, in roentgens, to bone is about the half of that

tolerated by soft tissues for 200 kV X-rays and may be no more than one-third for medium voltage and superficial X-rays (Spiers, 1951).

2. The affinity of osseous or associated tissue for a wide range of elements

It is not surprising that elements which are closely related to calcium are selectively retained by bone, for instance strontium and radium, nor that elements like sulphur that are probably built into the complex mucopolysaccharides may also be concentrated to some extent in bone matrix. There is in addition a large group of other elements which have been shown experimentally to be concentrated in the skeleton and which are spoken of as bone-seeking isotopes (Hamilton, 1947). They are yttrium, zirconium, cerium, thorium, plutonium and americum, lanthanum, praeseodymium, neodymium, promethium, samarium, europium, actinium, neptunium, curium. Some of them assume importance as products of uranium fission, others are used as industrial or research tools and may therefore all in the atomic age find their way into the human skeleton.

In considering the metabolic pathway of any element it must be remembered that many decay with the production of radioactive daughter products whose metabolic pathway may differ from that of the parent isotope. This is true in particular of mesothorium among the bone-seeking elements and has complicated the picture of mesothorium poisoning (Aub *et al.*, 1952). The behaviour in bone of the elements related to calcium, particularly strontium and radium appears to follow the same pattern, qualitatively at least, as calcium, and will be discussed in relation to the methods of bone growth. The behaviour of the other group is little understood in physiological terms and can only be described.

The deposition of bone-seeking isotopes other than calcium, strontium and radium. There is some evidence that the deposition of the group of bone-seeking isotopes other than those related to calcium follows the same pattern (Hamilton, 1947; Scott, Axelrod and Hamilton, 1949; Scott, Copp, Axelrod and Hamilton, 1948; Scott, Axelrod, Fisher, Crowley and Hamilton, 1948).

The behaviour of yttrium has been studied in the greatest detail and for the present may be taken as representative of this group, though further work may show that these elements show important differences in their metabolic pathway. The total amount of yttrium retained by the skeleton is less than the total amount of strontium that may be retained if given in large quantities, but the bone is more avid for small quantities, i e. it appears to be taken up from lower concentrations in the blood (Macdonald *et al.*, 1952; Rayner, Tutt and Vaughan, 1953). Of a tracer dose of ^{91}Y given to a young rabbit 70 per cent is retained in the skeleton eight weeks later, while an older rabbit retains almost as much (Rayner *et al.*, 1953). A puppy retains over 90 per cent of a tracer dose seven days later.

A study of both macroscopic and microscopic autoradiographs prepared from the bones of animals killed at different times after an injection shows extremely heavy concentration of ^{91}Y in the following sites in both young rabbits and young dogs directly after injection—(*i*) on the surface of vascularization on the growing side of the plate (Plate X, Figure 2); (*ii*) on the surface of periosteal bone in the metaphysis; (*iii*) on the surface of metaphyseal trabeculae; (*iv*) in association with small blood vessels throughout the cortical bone of the shaft (Plate XI, Figure 3); (*v*) on resorbing surfaces in building sites. These areas all stain with the periodic acid Schiff stain in varying degrees when preparations of undecalcified bone are used and in many cases are known to be sites of active bone resorption. Autoradiographs prepared from sections taken from animals killed some days after injection show a patchy distribution of the periosteal surface of the metaphysis and on the metaphyseal trabeculae. Resorption has occurred of the deposition in the region of the plate and there is often heavy deposition over cement lines due to formation of new bone which has overlaid the deposition on a bone surface present at the time of injection (Plate XII, Figure 4). No concentration of ^{91}Y is found in newly formed bone nor in osteoid seams in either normal puppies or puppies with rickets (Jowsey, Sissons and Vaughan, in the press). ^{91}Y appears to be concentrated on surfaces of bone where resorption is either active

or has just ceased and not in osteoid matrix as was originally suggested (Hamilton, 1947; Copp *et al.*, 1947)

The fact that bone tissue can become saturated with yttrium which does not apparently occur with strontium (Macdonald *et al.*, 1952) suggests that its uptake is not associated with the normal physiological mechanism of bone formation. It is known to form complexes extremely readily and it may be that it forms some complex with one or more element in bone or associated tissue which is relatively stable and therefore the high skeletal content persists. The skeletal burden can be greatly reduced by the administration of ethylene diamine tetra acetic acid, a powerful chelating agent (Tutt and Vaughan, 1953).

3. *The method of bone growth*

Bone grows in length by endochondrial calcification and it grows in transverse diameter by endosteal apposition in the region of the metaphysis and by periosteal apposition in the diaphysis. Throughout life constant remodelling of bone by resorption and apposition goes on in the corticalis. It is in these sites of active bone growth that radioactive isotopes related to calcium, such as strontium and radium are concentrated. In a young rabbit 70 per cent of the injected strontium is present in the skeleton within 10 minutes as shown both by chemical analysis and by study of autoradiographs, and it is largely present in areas of active bone growth, i.e beneath the epiphyseal plate, in endosteal bone, in the metaphysis, and in periosteal bone in the diaphysis (Plate XIII, Figure 5) and there it remains unless removed by the normal process of resorption. An autoradiograph of a longitudinal section of the tibia of the rabbit killed 18 months after injection of strontium shows that the bone containing radioactive isotope in the region of the plate has been resorbed, but that in the metaphyseal endosteal bone and diaphyseal periosteal bone is still in part present though its position in the bone is apparently lower owing to increased growth in length (Plate XIII, Figure 6).

This is shown even more clearly perhaps in the autoradiograph left on a thin cross-section of the tibia (Plate XIV, Figure 7).

Many of the cells that were originally exposed to irradiation where the isotope was first taken up are still exposed to radiation eighteen months later. In the case of older animals uptake in building sites associated with remodelling of bone is more important because it is in such areas that new bone is being formed and the radioactive isotope is incorporated, presumably in the new bone crystal. Microradiographs of cross-sections of the tibia of a monkey aged 4 years show many building sites with varying degrees of calcification in the surrounding bone (Plates XV, XVI, Figures 8, 10). Autoradiographs of the same sections show that the radioactive strontium given to the animal before death is concentrated in the areas of low calcification (Plates XV, XVI, Figures 9, 11). Subsequently new bone containing no isotope may be laid down on top so that in animals killed a long time after injection the isotope may appear unconnected with any building site (Owen, Jowsey and Vaughan, 1955). Radioactive calcium and radioactive phosphorus are concentrated in a similar way in growing bone (Leblond, Wilkinson, Bélanger and Robichon, 1950; Comar, Lotz and Boyd, 1952; Engfeldt, Engstrom and Zetterstrom, 1952; Lacroix, 1953). The amount of these elements present in non-growing bone is negligible. Examination of the bones of patients who have died of radium poisoning has also shown that radium is heavily concentrated in association with one Haversian system and not with another (Hoecker and Roofe, 1949, 1951) suggesting that the 'radium hot spot' described by Aub and his colleagues (Aub *et al.*, 1952) can be explained as due to the original deposit [illegible]

[illegible] growing bone (Engfeldt and Hyertquist, 1954; Bélanger, 1954; Lacroix, 1954; Kent *et al.*, in press), largely in the amino polysaccharides of the ground substance but possibly also in association with the bone crystal (Plate XVII, Figure 12) (Engfeldt and Hyertquist, 1954).

The fact that tumours or gross pathological change in bone may occur in areas showing little radioactivity at the time of death may be explained on theoretical grounds. Bone which contained radium at the time of ingestion may well have been

resorbed in the process of bone remodelling, but at the same time remodelling may not have included bone already damaged. Knowledge of bone physiology suggests there can be little migration of densely concentrated radium within the bone though possibly there may be uptake and secondary deposition (Jowsey *et al.*, 1953) following resorption into the blood stream in the normal process of remodelling.

Whatever isotope is considered whether in the calcium or the yttrium group, the important fact from the point of view of radiation damage is first that all bone-seeking elements appear to be concentrated in a patchy way in bone. An estimate of total skeletal content is no measure of the risk because the isotope is not evenly distributed. Secondly, once present in the bone the concentrated isotope is apparently only removed by the normal process of resorption. The processes of resorption and apposition in bone after the early period of growth are extremely slow and therefore even a small amount of radioactive element if it is a long-lived one such as radium or ^{90}Sr may continue to irradiate a small group of cells over many years. The original uptake in growing bone is rapid, the turnover due to resorption is slow. This character of bone growth may well in part at least account for the long latent period observed in the development of radiation damage (Looney, 1951; Aub *et al.*, 1952).

4. *Extensive crystal surfaces*

There has been considerable discussion in the literature about the part played by ionic exchange of bone-seeking elements upon the extensive crystal surfaces of the appatite lattice (Howard, 1953; Neuman and Neuman, 1953; Neuman and Weikel, 1954). The area of the lattice in an adult has been estimated to be from 2 to 15 acres (Armstrong, 1953).

Autoradiographic studies certainly show a slight diffuse reaction all over the bone surface in the case both of ^{90}Sr, ^{91}Y and ^{32}P which can be shown to be above background on grain counting. This reaction is heavier 24 hours after injection than it is 21 days after injection which favours the suggestion that isotope taken up on crystal surfaces forms a readily exchange-

able pool (Tomlin, Henry and Kon, 1953; Neuman and Weikel, 1954). Whether this indeed is so is extremely controversial (Maclean, 1953) and is not for discussion here. Interstitial uptake throughout bone, if it occurs, is so slight as to represent a minimum hazard. It is, however, possible that the mechanism of uptake in areas of active calcification, for instance in the building site, is dependent in part at least upon ionic exchange on crystal surfaces. It has been suggested (Engfeldt and Hyertquist, 1954) that in these areas the crystals of the bone salt are smaller and therefore take up calcium and strontium more readily. Some of the calcium can be shown by *in vitro* studies to be released from bone slices placed in normal saline particularly from the building site and endosteal and periosteal bone. As far as radiation injury is concerned it is the capacity of bone tissue, however old (Jowsey, Owen and Vaughan, 1955), to continue active growth and so promote localized concentration of certain elements and the ability of certain constituents of bone and associated tissues to concentrate other isotopes, that is significant.

IV. Relationship of Bone and Myeloid Tissue

The skeleton encloses the active and radio-sensitive blood forming marrow. The increased ionization that occurs in bone from any given dose of radiation is to some extent transferred to the adjacent marrow. The high skeletal burden of a radioactive element or heavy exposure of the skeleton to external radiation may result in severe blood dyscrasia. Bone lesions due to radiation characteristically develop slowly for physiological reasons as already discussed. The experimental animal or the patient will die of leukaemia or aplastic anaemia before bone lesions have had time to develop (Brues, 1949). The earliest deaths occurring in the radium dial-painting injury were due to profound anaemia (Hoffman, 1925; Martland *et al.*, 1925). Death from bone sarcoma occurred later (Martland and Humphries, 1929). The death-rate from leukaemia among the inhabitants of Hiroshima already exceeds that of the unexposed population (Molony and Lange, 1954). An increase in bone sarcomata in the coming years may well be expected.

resorbed in the process of bone remodelling, but at the same time remodelling may not have included bone already damaged. Knowledge of bone physiology suggests there can be little migration of densely concentrated radium within the bone though possibly there may be uptake and secondary deposition (Jowsey *et al.*, 1953) following resorption into the blood stream in the normal process of remodelling.

Whatever isotope is considered whether in the calcium or the yttrium group, the important fact from the point of view of radiation damage is first that all bone-seeking elements appear to be concentrated in a patchy way in bone. An estimate of total skeletal content is no measure of the risk because the isotope is not evenly distributed. Secondly, once present in the bone the concentrated isotope is apparently only removed by the normal process of resorption. The processes of resorption and apposition in bone after the early period of growth are extremely slow and therefore even a small amount of radioactive element if it is a long-lived one such as radium or ^{90}Sr may continue to irradiate a small group of cells over many years. The original uptake in growing bone is rapid, the turnover due to resorption is slow. This character of bone growth may well in part at least account for the long latent period observed in the development of radiation damage (Looney, 1951; Aub *et al.*, 1952).

4. *Extensive crystal surfaces*

There has been considerable discussion in the literature about the part played by ionic exchange of bone-seeking elements upon the extensive crystal surfaces of the appatite lattice (Howard, 1953; Neuman and Neuman, 1953; Neuman and Weikel, 1954). The area of the lattice in an adult has been estimated to be from 2 to 15 acres (Armstrong, 1953).

Autoradiographic studies certainly show a slight diffuse reaction all over the bone surface in the case both of ^{90}Sr, ^{91}Y and ^{32}P which can be shown to be above background on grain counting. This reaction is heavier 24 hours after injection than it is 21 days after injection which favours the suggestion that isotope taken up on crystal surfaces forms a readily exchange-

able pool (Tomlin, Henry and Kon, 1953; Neuman and Weikel, 1954). Whether this indeed is so is extremely controversial (Maclean, 1953) and is not for discussion here. Interstitial uptake throughout bone, if it occurs, is so slight as to represent a minimum hazard. It is, however, possible that the mechanism of uptake in areas of active calcification, for instance in the building site, is dependent in part at least upon ionic exchange on crystal surfaces. It has been suggested (Engfeldt and Hyertquist, 1954) that in these areas the crystals of the bone salt are smaller and therefore take up calcium and strontium more readily. Some of the calcium can be shown by *in vitro* studies to be released from bone slices placed in normal saline particularly from the building site and endosteal and periosteal bone. As far as radiation injury is concerned it is the capacity of bone tissue, however old (Jowsey, Owen and Vaughan, 1955), to continue active growth and so promote localized concentration of certain elements and the ability of certain constituents of bone and associated tissues to concentrate other isotopes, that is significant.

IV. Relationship of Bone and Myeloid Tissue

The skeleton encloses the active and radio-sensitive blood forming marrow. The increased ionization that occurs in bone from any given dose of radiation is to some extent transferred to the adjacent marrow. The high skeletal burden of a radioactive element or heavy exposure of the skeleton to external radiation may result in severe blood dyscrasia Bone lesions due to radiation characteristically develop slowly for physiological reasons as already discussed. The experimental animal or the patient will die of leukaemia or aplastic anaemia before bone lesions have had time to develop (Brues, 1949). The earliest deaths occurring in the radium dial-painting injury were due to profound anaemia (Hoffman, 1925; Martland *et al*, 1925). Death from bone sarcoma occurred later (Martland and Humphries, 1929). The death-rate from leukaemia among the inhabitants of Hiroshima already exceeds that of the unexposed population (Molony and Lange, 1954). An increase in bone sarcomata in the coming years may well be expected.

Though acute blood dyscrasia is characteristic of high radiation damage it is becoming apparent that patients with radiation dysplasia of bone, not necessarily malignant, that develops after a long latent period and a relatively small dose may also in the terminal stage develop a disordered blood picture. This may be diagnosed as multiple myelomatosis especially if associated with skull lesions. On the other hand it may be present as either acute myeloid leukaemia or leuco-erythroblastic anaemia. Since the bone change and the blood dyscrasia are known to be caused independently by radiation, it is tempting when they occur together to recognize a single aetiological factor rather than make a double diagnosis.

V. Conclusions

This brief summary of the available knowledge about the risks to the skeleton from both internal and external radiation raises many questions which require further investigation. Understanding of bone pathology due to radiation must await greater knowledge of normal bone physiology and of the fundamental effects of radiation on living cells. The following practical conclusions are, however, suggested:

(*i*) external radiation for the treatment of non-malignant conditions of the skeleton should not be used except in elderly patients;

(*ii*) long-lived bone-seeking radioactive isotopes should not be administered to human beings for diagnostic or therapeutic purposes;

(*iii*) the possibility of injury due to radiation must be considered in all cases of obscure bone disease;

(*iv*) the total radiation dose to the skeleton given over the years as a result of diagnostic or therapeutic procedures should be recorded for each individual.

Acknowledgements

The original work included in this paper owes much to my collaborators Barbara Kidman, Jenifer Jowsey, Barbara Rayner, Margaret Tutt and Maureen Owen I am also indebted to Dr. Ray Oliver for reading the manuscript and making many valuable sug-

gestions. To the Editor and Publishers of the *British Journal of Experimental Pathology* I am grateful for permission to include Figures 5 and 6

REFERENCES

AMPRINO, R. and BAIRATI, A. (1936). *Z. Zellforsch.* **24**, 439.
ARMSTRONG, W. D (1953) Josiah Macey Jr. Foundation· Metabolic Interrelations, 3rd Conference, p 39

BARDEN, S P. (1943). *Radiology*, **41**, 389
BECK, A. (1922). *Munch. med Wschr.* **69**, 623.
BÉLANGER, L. F. (1954). *Canad J. Biochem. Physiol.* **32**, 161

W.
and COLEY, B L. (1948). *Cancer, N.Y.* **1**, 3
COMAR, C. L , LOTZ, W. E. and BOYD, G A (1952). *Amer J. Anat* **90**, 113.
COPP, D. H , HAMILTON, J. G , JONES, D. C , THOMPSON, D M and CRAMER, C. (1951). Josiah Macey Jr. Foundation: Metabolic Interrelations, 3rd Conference, p. 226.
DALAND, E M. (1949) *Radiology*, **52**, 205
DALBY, R. G , JACOX, H. W. and MILLER, N. F (1936) *Amer J Obstet Gynec* **32**, 50
DUNLAP, C E , AUB, J C , EVANS, R D and HARRIS, R. S (1944) *Amer. J. Path* **20**, 1.
ENGFELDT, B , ENGSTROM, A. and ZETTERSTROM, R. (1952). *Biochem. biophys. Acta*, **8**, 375
ENGFELDT, B. and HYERTQUIST, S O (1954). *Acta path microbiol scand* **35**, 205
F

F
F
GATES, O. (1943). *Arch Path* **35**, 323
GRATZEK, F. R., HOLMSTROM, E. G. and RIGLER, L. G (1945), *Amer. J. Roentgenol* **53**, 62.
GRAY, L. H , MOTTRAM, J. C., READ, J. and SPEAR, F. C (1940) *Brit. J. Radiol.* **13**, 371.
GRAY, L H (1953) *Brit J Radiol.* **26**, 609.
HAMILTON, J. G. (1947). *Radiology*, **49**, 325.

HELLER, M (1948). In Bloom's *Histopathology of irradiation from external and internal sources*, National Nuclear Energy Series, division iv. McGraw-Hill, New York.

[illegible]

HOFFMAN, F. L. (1925). *J. Amer. med Ass.* 85, 961.

HOWARD, J. E. (1953). Josiah Macey Jr. Foundation. Metabolic Interrelations, 3rd Conference, p. 11.

JACKSON, F. S. (1953). 'Fibrogenesis *in vivo* and *in vitro*.' In Randall's *Nature and structure of collagen*. Butterworth, London, p. 140.

JARUSLAWSKY, W. (1929). *Zbl. Chir.* 56, 915.

JONES, A. (1953). *Brit J. Radiol.* 26, 273.

JOWSEY, J., OWEN, M. and VAUGHAN, J. (1953). *Brit J. exp. Path.* 34, 661.

JOWSEY, J., OWEN, M. and VAUGHAN, J. (1955). *Brit. J. exp. Path.* 36, 22.

JOWSEY, J, RAYNER, B., TUTT, M. and VAUGHAN, J. (1953). *Brit. J. exp. Path.* 34, 384.

JOWSEY, J., SISSONS, H. and VAUGHAN, J. *J. Nuclear Energy* (in the press).

KALAYJIAN, B. S. (1938). *Amer. J. Roentgenol.* 40, 383

KENT, P. W., JOWSEY, J., STEDDON, L. M, OLIVER, R. and VAUGHAN, J. *Biochem. J.* (in the press).

[illegible]

LACROIX, M P (1954). Radio isotope conference, 1, 134.

LEA, D. E (1946) *Actions of radiations on living cells.* Cambridge University Press, Cambridge.

LEBLOND, C. P, WILKINSON, G. W., BELANGER, L. F. and ROBICHON, J. (1950). *Amer J. Anat.* 86, 289

LISCO, H, FINKEL, M. P. and BRUES, A. M (1947). *Radiology*, 49, 361.

LOONEY, W B. (1951). Argonne National Laboratory, A N L. 4666.

LOONEY, W B and WOODRUFF, L. A (1953). *Arch. Path.* 56, 1.

MACDONALD, N. S., NUSBAUM, R. E, ALEXANDER, G. V., EZMIRLAIN, F, SPAIN, P and ROUNDS, D. E (1952). *J biol Chem.* 195, 837.

MARINELLI, L D., QUIMBY, E H. and HINE, G J. (1948). *Amer. J Roentgenol* 59, 260.

MARTLAND, H. S (1929). *J Amer med Ass* 92, 466 and 552.

MARTLAND, H S. (1931) *Amer J. Cancer.* 15, 2435.

MARTLAND, H S, CONLON, P and KNEF, J. P. (1925). *J. Amer med Ass.* 85, 1968.

MARTLAND, H. S and HUMPHRIES, R. E. (1929). *Arch. Path* 7, 406.

MCCRORIE, W D C (1950). *Brit J. Radiol.* 23, 587.

MCLEAN, F. C (1953). Josiah Macey Jr. Foundation. Metabolic Interrelations, 3rd Conference, p. 273.

MOLONY, W. C. and LANGE, R. D. (1954). *Blood*, **9**, 663

(1952). *Radiology*, **59**, 637.

NEUMAN, W. F. and NEUMAN, M. W. (1953). *Chem. Rev.* **53**, 1.

Recommendations of the International Commission on Radiological Units 1928, 1937.

SCOTT, K. G., AXELROD, D. J., FISHER, H., CROWLEY, F. J. and HAMILTON, J. G. (1948). *J. biol. Chem.* **176**, 283

SCOTT, K. G., AXELROD, D. J. and HAMILTON, J. G. (1949). *J. biol. Chem.* **177**, 325

SCOTT, K. G., COPP, D. H., AXELROD, D. J. and HAMILTON, J. G. (1948). *J. biol. Chem.* **175**, 691.

SLAUGHTER, D. P. (1942). *Amer. J. Roentgenol.* **48**, 201.

SMITHERS, D. W. and RHYS-LEWIS, R. D. S. (1945). *Brit. J. Radiol.* **18**, 359

SPIERS, F. W. (1946). *Brit. J. Radiol.* **19**, 52

SPIERS, F. W. (1949). *Brit. J. Radiol.* **22**, 521.

SPIERS, F. W. (1950). *Brit. J. Radiol.* **23**, 743.

SPIERS, F. W. (1951). *Brit. J. Radiol.* **24**, 365

SPIERS, F. W. (1953). *Brit. J. Radiol.* **26**, 296

STAMPFLI, W. P. and KERR, H. D. (1947). *Amer. J. Roentgenol.* **57**, 71.

41, 915

r. **7**, 235

in *The Physiology and Biochemistry of Bone*, Academic Press, New York.

WASSERMANN, F. (1952). Argonne National Laboratory, A.N.L. 4794

WHITEHOUSE, W. M. and LAMPE, I. (1953). *Amer. J. Roentgenol.* **70**, 721

XIII

Reaction of Enzymes to Injury

J. D. JUDAH

In this lecture I wish to consider injury purely as a subcellular phenomenon; that is to say, I am going to deal with the effects on cell function of a variety of noxious agents. Very little is known about the subject, for example, in the common condition of shock due to loss of circulating blood volume; the pathologists and physiologists have given us a very clear idea of the reactions of the organism as a whole but we know nothing at all of the exact way in which anhydraemia produces its destructive effects on the cells composing the various tissues. Nor do we know how it is that the condition becomes irreversible and unamenable to treatment. My colleagues Dr P. N. Magee and Dr. W. G. Spector (1952), in a masterly analysis of experimental anhydraemia, were unable to afford any explanation of this phenomenon. In collaboration with them, Dr. K. R. Rees and myself (Judah, Magee and Rees) undertook experiments in which we determined *in vitro* the metabolic activities of a number of tissues taken from animals in a state of extreme shock. We were unable to find any change. In addition, we studied by sensitive micro-methods the level of the nucleotide adenosine triphosphate (ATP) in the tissues of these animals We had reason to believe that this experiment might have served a useful purpose but in the event we found no significant change. We confessed defeat and left this problem for the moment.

I have started with this gloomy example to put you into the proper frame of mind and to make it clear that when we, as biochemists, tackle these great problems of pathology we are in

the position of blindfolded men in a coal cellar trying to thread a needle with black cotton. Only those who have shared our permanent state of bewilderment can appreciate the unhappy truth of this observation. Nevertheless, I am convinced that in the last analysis all injuries to cells by whatever means will one day be explicable in terms of injuries to enzymes.

Enzymic Structure of Cell

Before I go any further I should like to give you a brief account of the methods which we employ. Long ago when enzyme chemists were still talking about ferments it was recognized that the intracellular enzymes could be classified into those which were readily extractable after disruption of the cell and those which apparently needed an intact cell structure for their proper functioning. This distinction merely represents the confusion of a technical difficulty with a basic principle. As methods improved it became clear that all the enzyme functions of the cell could be studied *in vitro*. Recently, a rather more refined expression of this doctrine has been enunciated. It was found that intracellular enzymes could be divided into those which can easily be removed in a soluble state (e.g. all the enzymes of glycolysis) and those which adhere tenaciously to the cell debris, i.e. all the respiratory enzymes, and defy attempts to obtain them in solution in an active form. When this so-called debris was examined critically it appeared that it was capable of differentiation into several components. All the cell nuclei could be recovered from it and in addition a range of smaller particles were found which were thought to be the mitochondria, which are so abundant in the cytoplasm. The most recent of the methods for this fractionation of the cell into its parts is of an elegant simplicity (Hogeboom and others, 1948; Hogeboom and Schneider, 1950). The tissue (usually rat liver) is disintegrated in isotonic sucrose solution forming what we call a homogenate. This contains no intact cells but contains intact nuclei, what we are pleased to call the mitochondria and a variety of submicroscopic particles called microsomes. By centrifugation at low speed all the nuclei are thrown down, the fluid above them may be removed and centrifuged at a higher

XIII

Reaction of Enzymes to Injury

J. D. JUDAH

In this lecture I wish to consider injury purely as a subcellular phenomenon; that is to say, I am going to deal with the effects on cell function of a variety of noxious agents. Very little is known about the subject, for example, in the common condition of shock due to loss of circulating blood volume; the pathologists and physiologists have given us a very clear idea of the reactions of the organism as a whole but we know nothing at all of the exact way in which anhydraemia produces its destructive effects on the cells composing the various tissues. Nor do we know how it is that the condition becomes irreversible and unamenable to treatment. My colleagues Dr P. N. Magee and Dr. W. G. Spector (1952), in a masterly analysis of experimental anhydraemia, were unable to afford any explanation of this phenomenon. In collaboration with them, Dr. K. R. Rees and myself (Judah, Magee and Rees) undertook experiments in which we determined *in vitro* the metabolic activities of a number of tissues taken from animals in a state of extreme shock. We were unable to find any change. In addition, we studied by sensitive micro-methods the level of the nucleotide adenosine triphosphate (ATP) in the tissues of these animals. We had reason to believe that this experiment might have served a useful purpose but in the event we found no significant change. We confessed defeat and left this problem for the moment.

I have started with this gloomy example to put you into the proper frame of mind and to make it clear that when we, as biochemists, tackle these great problems of pathology we are in

compounds, the chief of these being a substance known as adenosine triphosphate (ATP). The energy which goes into the synthesis of this substance can be liberated; breakdown of ATP can also be regulated to the requirement of the system by the appropriate enzymes. Thus ATP and similar compounds represent a store of energy which can be depleted and recharged as required. This process of ATP synthesis during oxidation is known as oxidative phosphorylation.

I think you will all know that many enzymes need small molecular weight substances for their full activity. These coenzymes are usually rather loosely held and dissociate readily from their proteins. One of the characteristics of the mitochondrial system is that the component enzymes have their cofactors firmly attached and a surprising amount of damage must be done to the mitochondria before the coenzymes will diffuse away. In passing, I should like to point out that much of the confusion concerning enzymes which apparently need intact cell structure was due to ignorance of the functions of the coenzymes as well as methods of isolating the required system.

Effects of Carbon Tetrachloride

Now, having given you some idea of the enzymic structure of the cell, I should like to go back to my main theme.

I think it is worth while dividing up the methods by which a substance or set of conditions may attack enzyme systems into two main groups. In the first group I would place a direct attack on a particular enzyme. Into this category falls the well-known action of cyanide on cytochrome oxidase.

In the second main group the attack is not quite so much on a particular enzyme system as on the matrix upon which the enzyme is carried. One might see an analogy in the great naval battles of the last war where an attack on an aircraft carrier could be more effective than a series of blows aimed at the carrier's aircraft. I must say at once that this is a highly simplified conception because a bewildering series of interactions is possible, e.g an agent acting on a vital enzyme might so disorganize the subcellular structures which carry enzymes that they fail to function. Consider the enzymic synthesis of the

speed (9,000 × g). This brings down the mitochondria quantitatively, while further centrifugation (25,000–125,000 × g) may be used to bring down the other particles if required.

Determination of the distribution between these fractions of the respiratory enzymes showed that most of them reside in the mitochondria (Kennedy and Lehninger, 1949; Hogeboom and Schneider, 1951; Christie and Judah, 1953). Indeed it can be shown by the appropriate methods that the mitochondria contain all the enzymes of the tricarboxylic acid cycle and are capable of ozidizing pyruvate to CO_2 and H_2O without any external aid.

In contradistinction the nuclei seem to have a very poor selection of enzymes to work with. Even those enzymes known to be present are a hotchpotch which appear to have no known connected pattern of function.

The mitochondria consist of protein, pentose nucleic acid and phospholipide. They appear to be bounded by a semi-permeable membrane as demonstrated by the elegant experiments of my colleague Dr. W. G. Spector (1953), who determined the movement of electrolytes into and out of these particles. Dr. G. Christie and myself in collaboration with Dr. K. R. Rees have also obtained evidence relating to enzyme function which supports this view and I will deal with this point later.

Their enzymic constitution includes systems which will oxidize fatty acids, carry out transamination and amino-acid oxidation, and which will permit them to carry out the synthesis of their own protein, their own nucleic acid and, astonishingly, as shown by Kennedy (1953), they are capable of a total synthesis of their own phospholipid, i.e. given a fatty acid or glycerol or choline or phosphorus, these substances will be incorporated into the mitochondrial phospholipid. Such synthetic processes have to be energized and I need hardly say that the mitochondrial system is capable of utilizing the energy liberated during the oxidation of carbohydrates and fats (Kennedy and Lehninger, 1949; Judah and Williams-Ashman, 1951; Judah, 1951). The greater portion of the energy liberated by this oxidation is conserved and it now seems reasonable to believe that this happens by the formation of certain phosphate

compounds, the chief of these being a substance known as adenosine triphosphate (ATP). The energy which goes into the synthesis of this substance can be liberated; breakdown of ATP can also be regulated to the requirement of the system by the appropriate enzymes. Thus ATP and similar compounds represent a store of energy which can be depleted and recharged as required. This process of ATP synthesis during oxidation is known as oxidative phosphorylation.

I think you will all know that many enzymes need small molecular weight substances for their full activity. These coenzymes are usually rather loosely held and dissociate readily from their proteins. One of the characteristics of the mitochondrial system is that the component enzymes have their cofactors firmly attached and a surprising amount of damage must be done to the mitochondria before the coenzymes will diffuse away. In passing, I should like to point out that much of the confusion concerning enzymes which apparently need intact cell structure was due to ignorance of the functions of the coenzymes as well as methods of isolating the required system.

Effects of Carbon Tetrachloride

Now, having given you some idea of the enzymic structure of the cell, I should like to go back to my main theme.

I think it is worth while dividing up the methods by which a substance or set of conditions may attack enzyme systems into two main groups. In the first group I would place a direct attack on a particular enzyme. Into this category falls the well-known action of cyanide on cytochrome oxidase.

In the second main group the attack is not quite so much on a particular enzyme system as on the matrix upon which the enzyme is carried. One might see an analogy in the great naval battles of the last war where an attack on an aircraft carrier could be more effective than a series of blows aimed at the carrier's aircraft. I must say at once that this is a highly simplified conception because a bewildering series of interactions is possible, e.g an agent acting on a vital enzyme might so disorganize the subcellular structures which carry enzymes that they fail to function. Consider the enzymic synthesis of the

phospholipide molecule; five components are strung together to make a vital fraction of the mitochondrial structure. Failure to introduce one of these components into its correct place will result in breakdown of the complex mitochondrial functions that we have already considered.

In another case, the attack may deprive a given enzyme of its coenzyme. This in turn could lead to the consequences that I have already mentioned. Such an attack could be mediated by a structural change in the mitochondria. So that you see that the concept of the aircraft carrier represents reduction to absurd simplicity. It has however been of use to us and I will illustrate now the manner in which we came to formulate this idea.

Carbon tetrachloride is a substance which has long interested our department, and two years ago we decided that we might be able to find out how it worked to produce its well-known effect on the liver. My colleagues Drs. K. Rees and G. Christie (Christie and Judah, 1954) collaborated with me in what we hoped would be an essay in chemical pathology. As the problem bristled with difficulties, we decided that a straightforward attack would probably pay best, in that failure would rapidly become apparent and we would then be able to abandon the project without loss of time and energy. In parenthesis I might observe that it is just as important to know when to stop as when to press on with such work. We therefore poisoned large numbers of rats with carbon tetrachloride and examined their livers *in vitro*. The first results of our attempt were to plunge us into utter confusion. We got variable changes and the enzyme experiments were either negative or inconsistent. The reason for this was ignorance Dr. K. K. Cheng, of the M.R.C. Toxicology Unit, told us that we should give the poison by mouth and not by injection as we had been doing; he also observed that our doses were inadequate and that we should standardize our methods and use animals which were of the same strain, sex and age. As all this was already knowledge, and Dr. Cheng had done a good deal of work in our own laboratory on the subject, we felt suitably humble, and started again. In the first instance we studied the respiration of liver slices suspended in Ringer solution. This tells one how the metabolism of the whole

cell is affected, and we found that some 15–20 hours after poisoning with CCl_4 the oxygen uptake of the cells was grossly reduced. The next step was to investigate the behaviour of cell-free preparations of poisoned livers, since it is clear that experiments with whole cells are not capable of telling what system, either enzymologically or topographically, is affected. Experiments with homogenates revealed that the oxidation of several substrates was grossly reduced; the effects were maximal some 15 hours after poisoning. To be more precise, we found that the oxidation of fatty acids was inhibited completely; that the oxidation of pyruvate was severely reduced; that the oxidation of citrate was feeble, while in contrast, the oxidation of succinate and α-ketoglutarate was relatively normal.

As these enzymes are heavily represented in the mitochondria, it was an obvious next step to isolate these particles from poisoned livers and see how they behaved When this was done, the results were in good accordance with those obtained from the cruder experiments with homogenates, but we observed that the changes were more severe and in fact that the oxidation of the amino-acid, L-glutamic acid, which had been relatively unaffected in the whole homogenate, was, in the mitochondrial preparation, very greatly reduced. Much thought about this made it clear that a difference between the homogenate and the mitochondrial preparation obtained from it lay in the time which elapsed before the enzyme system was tested. In the case of the homogenate this was only a few minutes, while the mitochondria had to be isolated, which took 60 minutes or more. Investigation revealed that this ageing process was in fact responsible for the loss of activity of the mitochondrial preparation toward glutamic acid. As normal mitochondria do not show such a loss, it was clear that we had stumbled upon a mitochondrial change. The susceptibility of mitochondria from poisoned livers to ageing *in vitro* was extensively investigated. We studied them at various times after poisoning and we tested a variety of substrates. The following points emerged. (1) different substrates were affected by ageing to different degrees; (2) the ageing effect varied with the time after poisoning; (3) the oxidation of citrate was first hit some 5–10 hours after

poisoning, and the ageing phenomenon was observed some 30 minutes after preparation of the mitochondria; at this time, the oxidation of malate was susceptible only after some 3 hours of ageing; the other substrate oxidations were unaffected; (4) at 10–15 hours after poisoning the trend was more marked: Malate oxidation now showed an ageing effect within 30 minutes, while the oxidation of glutamate which had previously been resistant was also affected, but only some 60–90 minutes after preparation. And so on. In other words, a progressive mitochondrial lesion had been uncovered. The nature of the lesion was next studied. We found that the enzyme changes which I have just described were reversible by the addition of coenzyme I to the Warburg flasks; as the mitochondria normally hold fast to their coenzymes, it seemed to us that the carbon tetrachloride had had the effect of making them leaky and thus allowing the small cofactor molecules to diffuse away. We confirmed this theory by making use of the elegant spectrophotometric method for studying the action of malic dehydrogenase. In this method, the enzyme, its substrate (L-malic acid) and its coenzyme are placed in the cell of a U–V spectrophotometer. Oxidation of the malic occurs with the simultaneous reduction of the coenzyme, and it is known that the reduced form of this molecule has a pronounced absorption of the U–V at a wavelength of 340 mμ. It is therefore possible by following the increase in absorption at this wavelength to determine the rate of the enzyme reaction. Christie and I had found in some other work that the rate of reduction of coenzyme I by normal rat-liver mitochondria in the presence of L-malic acid was extremely low, and we had also found that if the mitochondria were burst open by suspending them in distilled water, then the rate of reduction increased by a factor of 7. We had concluded on the basis of other information in our possession that a permeability barrier to CoI existed in normal rat-liver mitochondria. In the case of carbon tetrachloride injury, then, we should find an increased rate of CoI reduction, since if the molecule were able to diffuse away more readily, it followed that it should also gain access more readily. This was, in fact, what we found.

The evidence, therefore, pointed to an attack on the mitochondria and it seemed that carbon tetrachloride did not damage a specific enzyme, but brought about a structural alteration. Several other pieces of information all pointed in this direction: we were able to show that CCl_4 was able to bring about its effects on the mitochondrial system *in vitro*. When a normal liver homogenate was preincubated with the poison for several hours, and its mitochondria were then isolated, they showed the characteristic changes in enzyme activity. Furthermore, we were able to isolate from poisoned livers structures which we had reason to believe were damaged mitochondria. This was done as follows. Dr. Christie had observed the accumulation of cytoplasmic globules after carbon tetrachloride poisoning; these globules varied in diameter from 1–5μ and were sometimes larger still. We also found that in the homogenates prepared from damaged livers there was an accumulation of these globules. They were identified by differential staining both *in vivo* and *in vitro*. We isolated them for study by a suitable modification of the centrifugal procedure and by chemical and enzymatic investigation were able to obtain reasonable evidence that they were derived from the mitochondria. We therefore present carbon tetrachloride injury as an example of a direct physical attack on the subcellular structure and believe that this is the first time that such a mechanism has been given reasonable experimental support. In conclusion, I should like to mention the well-known effect of choline in ameliorating the action of carbon tetrachloride. We found that choline not only postponed the histological evidence of damage, but also had a similar effect on the enzyme changes Following this up, we determined the rate of phospholipid synthesis in isolated mitochondria from normal and poisoned livers, by the use of radioactive phosphorus No change was found in the rate of synthesis, a finding that we are unable to explain, and which is rather a fly in the smooth ointment which I have just dispensed. In fact, it is startling that grossly damaged mitochondria are capable of carrying out this complex synthesis Another curious thing is the activity of another complicated function which is not impaired, i.e. oxidative phos-

phorylation. We found this going strong at a point where severe enzyme change was evident. On this unsatisfactory but characteristic note, I should like to leave the problem and consider an investigation which we have under way at the present time.

COPPER DEFICIENCY

The classical studies of Sir Rudolph Peters (1953) on thiamine deficiency must by now be widely known. During this work he was not only able to show the point of enzyme failure in the deficiency of vitamin B_1, but also postulated the theory of the biochemical lesion. It is hard to estimate the debt which we owe to Peters and his school. A good deal of their work has been devoted to problems of the type I am here considering, and it is obvious to me that any success which we may achieve has been built on the foundations laid by them. At all events, I mean to speak now of experiments with a deficiency, that of copper, which is engaging our attention. Dr. Cliff Gallagher, of the McMaster Laboratory in Sydney has been the mainspring of this tedious investigation (Gallagher, Judah and Rees, 1955 a and b). As part of a programme with the general idea of investigating demyelinating disease, we decided to look into the enzymatic basis of copper deficiency. Our experimental animal is the rat, and while we are well aware that this animal does not show demyelination due to this particular deficiency, we assume that the general mechanism must be the same, whatever the tissue studied. In any case, we are also spreading our net to other species, but the results of these experiments are not yet known.

I will not dwell on the sufferings of Gallagher in trying to induce copper deficiency in rats, but after considerable trouble the typical deficiency syndrome made its appearance: the black and white rats used in the experiments lost weight; their pigmentation decreased so that they looked like faded blondes; their haemoglobin levels fell from a normal 10 gm. to about 4 gm. per 100 ml. of whole blood; and they failed to grow. Basing our attack on our experiences in the carbon tetrachloride investigation, we undertook a rapid survey of intracellular respiratory enzymes. Two main types of change were

observed. In the first place we found mitochondrial changes of the type found in carbon tetrachloride poisoning, but these were far less severe, unless the animals were on the point of death; also they were more specific. Citrate and malate oxidation are the only systems affected. The other change is quite a different matter and it consists in a powerful reduction in the activity of the cytochrome oxidase system. This is so marked that a reduction of some 70 per cent is found in rats with moderate deficiencies, whose haemoglobin levels are still around 7 gm. per 100 ml. From this observation several problems arise. First what is the nature of the deficit of cytochrome oxidase? Is it due to lack of some essential part of the system, e.g. a coenzyme? or is it due to a highly specific mitochondrial structural change which disrupts the cytochrome oxidase system only? Such changes are known and the recent work of McFarlane and Datta on the enzyme disturbances following incubation of rat-liver mitochondria with the *Clostridium welchii* lecithinase show that spatial disorientations are likely to occur. They were able to show that the oxidation of succinate was grossly inhibited (order of 90 per cent) while the cytochrome oxidase was reduced by some 50 per cent. The succinoxidase system is a complex, the two main components being succinic dehydrogenase, which carries out the actual oxidation of the substrate and the cytochrome oxidase system which catalyses the hydrogen transfer to atmospheric oxygen. Now it was found that the dehydrogenase in the lecithinase treated mitochondria was to all intents within normal limits, which means that destruction of the succinoxidase exceeded that of its component parts. This observation has been interpreted to mean that a spatial disorientation has occurred, and the nature of the lecithinase attack certainly makes this seem reasonable. To return to the copper problem, the choice between structural attack and coenzyme deficit will obviously have to be resolved, and to do this a direct experiment is necessary. We are now considering this problem in some detail. The second problem that we have to consider is whether the observed enzyme changes are responsible for the state of the animals on the deficient diet, and this is not easily resolved. Dr. Gallagher is of the opinion that the

whole syndrome could be so explicable; while I feel that our evidence is not complete. To explain, I should say that the cytochrome oxidase system is present in great excess in the cells. It is found for example, that reductions in the enzyme of some 70 to 80 per cent do not show up when it is catalysing the terminal oxidation of a cell-free preparation and it certainly does not show up in measurements on whole cells, as we have found. Thus a deficit estimated by methods which are designed to measure the activity of the single enzyme may well fall into the large reserve capacity of the system and be lost to view when the activity of the whole cell is being determined. Dr. Gallagher's view is that the restriction of cytochrome oxidase places a check on the growth of the organism, in particular—and we now talk of the rat—on the development of its blood haemoglobin.

Another possibility is that copper deficiency results in a failure of haem synthesis. We determined the activity of catalase, a haem enzyme, in the livers of our deficient rats and found no change at a time when cytochrome oxidase was down by some 80 per cent.

Evidently we have some hard thinking to do, and while this sort of work gives rise to multiple publications, I do not feel that this is a sufficient reason for the expenditure of time and effort. Nevertheless, the copper problem is a good example of the difficult task in front of us, in particular if the object is to get a reasonable idea of the mechanism of the syndrome, rather than to make a series of inconclusive surveys of the subject. I have not the least idea whether we have the wit to solve the problem, or even whether it is amenable to the treatment that we are giving it, and I would be a fool if I let it rest for the time being by saying that further work will give us the answer.

Enzymes of Human Liver

I have spent so long discussing the work of our own department, not because I think it is the only example of its kind, or because it is of outstanding importance, but simply out of familiarity with the thoughts and methods which have led to it. I now turn to work which I know of only through the published literature,

and which is particularly relevant to my general topic. The problem of human liver disease is one which has had much attention from clinicians and pathologists, but the work of Waterlow (1953), who has studied the enzymic constitution of human liver in biopsy material, seems to me to be singular in the freedom with which difficult methods have been applied under awkward conditions. The application of enzyme techniques to liver biopsies is complicated by the small amount of tissue available. Waterlow has made use of the Cartesian diver method for his experiments; in this method very small masses of tissue suffice for a reasonably accurate determination of enzymes along the same lines that we ourselves have followed, but for which about 1 mg. wet weight of liver is needed instead of the unlimited amounts which we can use in our experiments. Perhaps I should explain how the diver works. It is a small vessel whose gas volume and weight are so related that it will just float in the medium chosen for it; this medium is contained in a flotation chamber, which in turn is contained in a thermostat. The flotation chamber is connected to a manometer and a pressure regulator, and the conditions are so arranged that the diver at equilibrium is near the middle of the flotation chamber. The tissue whose respiration is to be measured is placed in the diver; if it absorbs oxygen, the gas volume of the system will decrease, and it will tend to sink. Negative pressure applied to the flotation vessels will then bring it back. By watching the diver through a horizontal microscope, it can be kept more or less in its equilibrium position and the amount of negative pressure necessary to keep it there will be a measure of the oxygen uptake; the manometer reading will of course provide this information I rather hesitate to express an opinion of this work of Waterlow, as it is obviously in progress, but from what I have read, I do not think he would disagree with me when I say that the correlation of pathological change and enzyme change is not good. This is to me disappointing, because I had hoped that some clear-cut picture would emerge. Nevertheless, this is a brave piece of work, and it is to be hoped that it will eventually have a good result.

In passing, I should mention that Waterlow's figures for the

activity of some of the intracellular enzymes of human liver present striking contrasts to what we find in the rat. Thus cytochrome oxidase is only present to the extent of about 12 per cent of that found in rat and succinoxidase shows some 2·5 per cent of the activity in human liver as compared to the rat. Whether this is due to some peculiarity of the system in humans so that *in vitro* study gives a false impression of the enzyme activity is not clear, but I should be interested to know whether any experiments have been carried out on this point. It would also be interesting to have an investigation along the lines of the carbon tetrachloride experiments that I have mentioned, but I do not know whether the technical difficulties could be overcome. From what I read, it seems to me that the enzymes studied by Waterlow, i.e. cytochrome oxidase, succinoxidase, lactic dehydrogenase, cholinesterase and transaminase might not be those which are affected in anything but a secondary manner.

I hope that I do not give the impression of being patronizing, because it would be an erroneous one. I can appreciate very well the difficulties of the work both on account of the subject and the place in which it was done, and I am full of admiration for it.

I do not think that any summary of my theme is possible. We hope that this kind of chemical pathology will give some insight into the fundamental mechanism of disease. We have done little enough to wish to do more, and not enough to feel either that we are wasting our time or that we are entering into a golden era of wonderful results and easy papers In biochemistry, there is no point in being ahead of your time; to be so means that your ideas have outstripped your methods and are therefore useless except as dreams Methods are the lifeblood of our science: anyone with a little common sense and imagination can supply the ideas.

REFERENCES

CHRISTIE, G. S and JUDAH, J. D. (1953). *Proc roy Soc.*, B, **141**, 420
CHRISTIE, G. S and JUDAH, J. D. (1954). *Proc roy. Soc.*, B, **142**, 241.
GALLAGHER, C H., JUDAH, J. D and REES, K. R. (1956a and 1956b). *Proc. roy. Soc.*, B. In press
HOGEBOOM, G. H., SCHNEIDER, W. C and PALLADE, G E. (1948). *J. biol. Chem.* **172**, 619.
HOGEBOOM, G. H and SCHNEIDER, W. C (1950). *J. biol. Chem.* **186**, 417
HOGEBOOM, G. H and SCHNEIDER, W. C (1951). *Cancer Res.* **11**, 1.
JUDAH, J. D, MAGEE, P. N. and REES, K. R. Unpublished work
JUDAH, J. D and WILLIAMS-ASHMAN, H. G. (1951) *Biochem J.* **48**, 33
JUDAH, J D. (1951) *Biochem J.* **49**, 271
KENNEDY, E P. (1953). *J. biol. Chem* **201**, 399.
KENNEDY, E. P and LEHNINGER, A. L. (1949). *J. biol Chem.* **179**, 957.
MAGEE, P. N. and SPECTOR, W. G. (1952) *Proc. roy. Soc.*, B, **139**, 584
PETERS, R. A. (1953). *Brit. med Bull.* **9**, 116
SPECTOR, W. G. (1953) *Proc roy. Soc.*, B, **141**, 268
WATERLOW, J. (1953) *Liver Injury*, Josiah Macy, Jr. Foundation, New York, p. 72.

XIV

The Effects of Alcohol

J. H. GADDUM

THE fascinating pharmacological effects which can be produced by drinking fermented liquors must have been discovered thousands of times in very early days by experimenters all over the world working with a great variety of raw materials. It would be foolish to suggest that all these effects were due to ethyl alcohol, but a certain proportion of them undoubtedly were. The isolation and synthesis of this active principle and the discovery that it was present in varying amounts in so many different drinkable liquids simplified the pharmacological problem and diverted attention away from the many other substances which contribute to the enjoyment of the connoisseur.

The study of the effects of alcohol has proved attractive to pharmacologists and others, some of whom have acquired a reputation for consuming more than was strictly necessary for their investigations. Apart from the intrinsic interest of the subject, generous grants from public bodies have stimulated research, and a large number of original papers, reviews, monographs and other books have been written about alcohol. There is actually a *Quarterly Journal of Studies on Alcohol*.

In concentrations such as 2 or 3 per cent alcohol temporarily stops most of the manifestations of life. The movements of amoebae are slowed and tadpoles are anaesthetized. Experiments of this type have been used to compare different alcohols with one another, and it has always been found that the activity increases steadily with the size of the molecule, so that ordinary ethyl alcohol is almost the least active of them all. This effect

is reversible; when the alcohol is removed the animals recover, provided they have not been exposed too long. Much higher concentrations are needed to kill bacteria and it has been found that the maximum disinfectant action is produced by a concentration of 70 per cent.

A concentration of 2 per cent of ethyl alcohol inhibits isolated tissues such as heart or nerves, but such a concentration is never attained *in vivo* because the animal dies first, owing to general anaesthesia and depression of the respiratory centre. The action of alcohol on the central nervous system overshadows all its other actions and will form the main theme of this lecture, but it will be convenient to deal first with one or two secondary questions.

Absorption and Fate

When alcohol is taken by the mouth it is rapidly absorbed and distributed evenly through all the water in the body; including inside the cells. This fact can be used to make a rough estimate of the amount of water in the body; or if it is assumed that water forms four-fifths of our bodies, the concentration produced by a given dose of alcohol can be calculated. One ml. of alcohol weighs about 0·8 g. One kg. of body contains about 800 g of water. Therefore 1 ml. per kg. produces a concentration of 0·8 g. per 800 g. or 1 g. per kg. One part per thousand by volume makes a concentration of one part per thousand by weight.

Alcohol is excreted in the breath and urine and the concentration in the urine is about 33 per cent greater than the concentration in the blood. This may be just because urine contains more water than the blood does.

If urinary excretion was the only path by which alcohol disappeared from the body, the process would be a very slow one. The amount of urine excreted in 24 hours is generally between 1 and 2 litres and since the concentrations in urine and body water are about the same, the rate of clearance by this route must be about 1 or 2 litres of body water per 24 hours. Since there is normally something like 50 litres of water in the body, it would take many days to get sober if this were the only way.

Alcohol is oxidized in the body to acetaldehyde and then to water and carbon dioxide. This process is more or less independent of the concentration of alcohol and of the needs of the body, and proceeds at a steady rate of about 7 g. of alcohol per hour. This corresponds to the production of about 1,200 calories per 24 hours. It is clear that man cannot live long on alcohol alone.

Systemic Effects

The main effect of alcohol on the circulation is an increase of the blood flow through the skin at the expense of other tissues, so that the skin gets warmer and the blood gets cooler. It increases comfort, but diminishes resistance, and may thus benefit the man who has come in but not the man who is going out into the cold.

Alcohol has a diuretic effect greater than that of an equal volume of water. There has been doubt about this in the past, but the facts now seem to be clearly established. Alcohol causes diuresis not only in medical students, but also in rats, and it seems to do this by inhibiting the release of vasopressin by the posterior pituitary. The kidneys are normally subject to the antidiuretic action of this substance and the excretion of urine increases when the liberation of vasopressin is inhibited. These facts have been applied in the assay of vasopressin; rats receive alcohol, which makes them dead drunk and inhibits their pituitaries. They are then particularly sensitive to the antidiuretic effect of injections.

The restorative effect of the brandy which is sometimes given to those who have fainted is largely due to a reflex arising from the local action of the brandy on the throat, which stimulates the medulla. Similar effects are produced by sal volatile, or a douche of cold water or strong emotion or a slap on the face.

The action of alcohol on the central nervous system is similar to that produced by general anaesthetics and the fundamental explanation of its mechanism is still a matter of controversy. A number of ingenious theories have been propounded, but no one really knows how it works. It is, however, generally agreed that the main effects of alcohol are depressant and that

most of the apparent stimulant effects are due to depression of the highest centres in the brain which normally exert a restraining influence on the simple impulses and passions, which result from the thoughtless activities of the basal nuclei and the hypothalamus.

The actions of alcohol on human beings are sometimes divided into four stages. The first stage was described by Horace when he sang the praises of wine in words which I will give you in an English translation.

'What wonders wine does. It discloses secrets, confirms our hopes, thrusts the coward forth to battle, eases the anxious mind of its burden, and instructs in arts. Whom has not the cheerful glass made eloquent? Whom has it not freed from the pinch of poverty?'

Falstaff (1405) has also described the same clinical condition from another point of view and adds 'If I had a thousand sons, the first human principle I would teach them should be to forswear thin potations, and to addict themselves to sack.'

The man who has drunk wisely, feels pleased with himself and full of confidence, and may find that he can do difficult things better than he expected. Alcohol removes the inhibitions which may prevent a man from doing his best, but in other respects its effects on the brain are bad. It diminishes our powers of noticing things, slows our reflexes, upsets our balance, prevents accurate movements and slows our thought; there is scientific evidence of all these things.

In the second stage of alcoholic intoxication a man becomes drunk and disorderly. He is generally unsteady on his feet; his emotions are unstable and his actions unpredictable. It is clear to all and sundry that he has had too much and he may either pull himself together and do what he can to hide the facts, or let himself go and do real damage.

In the third stage of alcohol a man is dead drunk. Noah seems to have reached this stage when he was found by his second son Ham lying naked in his tent. Ham did not behave with sufficient tact and Noah put a curse upon him and upon his descendants.

Tests of Intoxication

The first scientific study of the effect of alcohol on the human brain was published in the *Journal of Physiology* by Warren in 1887, but the first really satisfactory investigations were described in a book by Kraepelin in 1892. He studied its effect on various simple psychological tests involving such things as the manipulation of words, mental arithmetic and guessing the time and found that the efficiency of the brain was generally decreased, but there was often a temporary increase in the performance of his subjects on the dynamometer. It has been suggested that this was due to inhibition of the reflexes which normally protect the muscles from doing too much work.

During the last sixty years many people have followed in Kraepelin's footsteps and many different tests have been developed. These tests have involved such things as typewriting, rifle practice, obedience to signals, sensory activity and discrimination, as well as lower reflexes such as knee jerks.

After small doses of alcohol most people approach such tests with confidence and are convinced that their efficiency is greater than normal, but the actual results show that they are wrong and that the effect of alcohol is a decrease in their score.

Let us consider in some detail the results of a careful and extensive investigation by Goldberg (1943) of the effects of alcohol on human beings. He used two tests of sensory functions, two tests of motor functions and two tests of psychological functions and got similar results with all six tests. The dose was 120–340 ml. of brandy (which is perhaps equivalent to 2–5 large whiskies) and the blood alcohol rose to about 1 part per thousand.

His first sensory test depends on the fusion frequency for flickering light. This is easier to detect when the light is bright and it is therefore possible to measure either the quickest detectable flicker with a given brightness, or the brightness just [illegible] frequency. Both [illegible] of the brain was [illegible] other tests) curves showing the effect at different times after the drink were very much the same as curves showing the blood-alcohol at different times after the drink.

The second test depended on the blink produced by a puff of air on the cornea. This puff came from a standard jet at a standard distance from the eye, and the pressure of the air behind the jet was increased until a blink occurred. After alcohol higher pressures were required and the effect of the alcohol was measured by the change in the threshold pressure.

The first test of motor functions was the Romberg test. An electric light was fixed on the top of an extendable bar which was attached to a frame on the shoulders of the subject. A miniature camera, pointing directly downwards from above, recorded the movements of this electric light, which was always 2 metres from the floor so that small men should have no advantage. The shutter was opened for fifteen seconds and the record was obtained in the form of an irregular wavy line on the film. This wavy line was enlarged and straight lines were ruled to enclose the whole pattern in one area, which was measured. This area was increased by alcohol and the effect rose and fell as the blood alcohol rose and fell. The ordinary Romberg test was carried out with the feet together. Each subject was also tested with one foot in front of the other and this was called the modified Romberg test. In this position the movements were of course greater—but the effect of alcohol was greater too.

The second test of motor functions was based on the clinical test in which the patient closes his eyes and then tries to make his two first fingers meet. On one finger there was a thimble with a sharp point, and on the other there was a disc 8 8 cm. in diameter with paper attached. The subject closed his eyes and then tried to bring his fingers together from a distance of 40–50 cm., so that he made a mark on the paper. This process was repeated fifty times in 1½ minutes, in time with a metronome. The area covered by the marks on the paper was enclosed by straight lines and measured. Under the influence of alcohol the area became larger.

The first psychological test was called the subtraction test. The subject was asked to start with some number between 99 and 104 and to subtract 7 and then subtract 7 again and so on until there was nothing left. The total time that it took to do this was used as a measure of the result. After a little practice

Tests of Intoxication

The first scientific study of the effect of alcohol on the human brain was published in the *Journal of Physiology* by Warren in 1887, but the first really satisfactory investigations were described in a book by Kraepelin in 1892. He studied its effect on various simple psychological tests involving such things as the manipulation of words, mental arithmetic and guessing the time and found that the efficiency of the brain was generally decreased, but there was often a temporary increase in the performance of his subjects on the dynamometer. It has been suggested that this was due to inhibition of the reflexes which normally protect the muscles from doing too much work.

During the last sixty years many people have followed in Kraepelin's footsteps and many different tests have been developed. These tests have involved such things as typewriting, rifle practice, obedience to signals, sensory activity and discrimination, as well as lower reflexes such as knee jerks.

After small doses of alcohol most people approach such tests with confidence and are convinced that their efficiency is greater than normal, but the actual results show that they are wrong and that the effect of alcohol is a decrease in their score.

Let us consider in some detail the results of a careful and extensive investigation by Goldberg (1943) of the effects of alcohol on human beings. He used two tests of sensory functions, two tests of motor functions and two tests of psychological functions and got similar results with all six tests. The dose was 120–340 ml. of brandy (which is perhaps equivalent to 2–5 large whiskies) and the blood alcohol rose to about 1 part per thousand.

His first sensory test depends on the fusion frequency for flickering light. This is easier to detect when the light is bright and it is therefore possible to measure either the quickest detectable flicker with a given brightness, or the brightness just sufficient to cause a detectable flicker at a given frequency. Both methods gave similar results. The efficiency of the brain was diminished, and in this test (and in all the other tests) curves showing the effect at different times after the drink were very much the same as curves showing the blood-alcohol at different times after the drink.

some were heavy drinkers and all the tests showed clear differences between these three groups. The average threshold concentration was about 0·33 parts per thousand for abstainers and about 2½ times as much as this for heavy drinkers. There was no difference between the concentrations produced by a given dose, but there was quite a large difference between the effects produced by a given concentration. A given degree of intoxication would cost the average heavy drinker about 2·5 times as much as it would cost the average abstainer. The mechanism of this tolerance is unknown, but it clearly does not depend on any change in the rate at which alcohol is inactivated in the body.

Accidents due to Alcohol

When an accident has occurred on the road doctors are sometimes asked to decide whether the driver was 'under the influence of drink or a drug to such an extent as to be incapable of having proper control of the vehicle'. If this is shown to be the case, then he has committed an offence under the Road Traffic Act (1930). A special committee of the British Medical Association has recently prepared a report on 'The Recognition of Intoxication' (B.M.A., 1954) which gives much useful information for the guidance of doctors in such cases. This report gives a model scheme of medical examination with details of the most important questions to be asked and observations to be made. Simple tests of memory, mental alertness, writing, muscular coordination, etc. are described, but tongue-twisters are condemned as undesirable. The section devoted to the differential diagnosis gives an impressive list of pathological states simulating alcoholic intoxication. These include such things as head injuries, hypoglycaemia, uraemia, various neurological and psychiatric conditions, high fever, and poisoning by carbon monoxide, barbiturates, antihistamines, morphine, atropine or hyoscine.

The committee recommends that estimations of the concentration of alcohol in the body fluids should be made more frequently in this country than is now the case. For this purpose it is necessary to secure a sample of blood, urine or expired air. Expired air is perhaps the easiest of these to obtain and various

this quantity became reasonably constant. In sober individuals errors were too rare to be worth considering; under the influence of alcohol the time increased and errors increased.

The second psychological test was known as the Bourdon test. The subject was given a printed page and asked to find as many as possible of each of three different letters and to mark them with three different signs—a circle for one letter, a cross for another and a sloping line for the third. He was told to work as quickly as possible and allowed 4 minutes. The performance during the first minute was poor, because the subjects were not accustomed to this kind of work, but it was found that after this time they had acquired sufficient practice and the results became nearly constant. Under the influence of alcohol the number of letters correctly marked was less than normal.

The results of all these six tests were calculated in the same way. The scores before and after alcohol were converted into logarithms and the difference between these two logarithms was taken as a measure of the effect of the alcohol. This mathematical device had two advantages; it reduced all the scores to the same scale and it led to the production of straight lines when the effect of alcohol was plotted against the concentration in the blood. This method of experimenting was really most convenient, since it made it possible to study the effects of a whole series of concentrations by giving a single large dose and making observations of the effects and concentrations at intervals during the time that the concentration was falling. Incidentally, it may be worth mentioning the curious fact that a given concentration has more effect when the blood-alcohol is rising than when it is falling.

All the tests gave similar results, but the ordinary Romberg test gave particularly good results and it may be calculated that it should be possible to determine the concentration of alcohol in the blood with a standard error less than 0·1 parts per thousand from the results of a Romberg test done by the method described here.

These tests were used by Goldberg to study the tolerance for alcohol which may be acquired by regular drinking. Some of the subjects were abstainers, some were moderate drinkers and

of any kind, either because they are afraid of the truth, or because they are afraid that the results of the tests may be misinterpreted in the courts, or for some other less logical reason. Let us consider, therefore, what conclusions can justifiably be drawn from the results of such tests.

It is clearly not possible to separate the drunk from the sober in terms of a single critical concentration of alcohol in the blood. The average heavy drinker can stand 2½ times as much as the average abstainer. In Jetter's (1938) data 90 per cent of persons with 250 mg./100 ml. of alcohol in their blood were judged intoxicated and only 10 per cent of persons with 50 mg./100 ml. The toughest 10 per cent could stand five times as much alcohol as the 10 per cent with the weakest heads. In countries such as ours, where the court has to decide whether the driver was incapable of having proper control of the vehicle, an estimate of the alcohol in blood or urine cannot outweigh all other evidence, but should be available when a decision is reached. It cannot prove that he was drunk, but it may prove that he had been drinking large amounts of alcohol and this knowledge should carry weight when all the evidence is considered For example, the B.M.A. committee estimate that if the urine-alcohol is 196 mg./100 ml. after beer the man must have had at least 4 pints and probably more. If he says he had less, he is a liar and if he behaved like someone who has had too much alcohol, then there is no real need to consider the theory that his behaviour was due to other causes, even if he is found to suffer from some disease which might have made him appear drunk without being so.

On the other hand, if the concentration is low in a sample collected soon after the accident, then alcohol can be exonerated from blame, and persons who believe themselves wrongly accused of drunken driving should be offered an opportunity of proving themselves innocent by having their blood or urine tested. In any case, if the doctor knows that there was little or no alcohol in the urine, then he must find some other cause for any symptoms he observes.

If estimates are made in samples from everyone who is involved in an accident, they may help to show who was really

machines have been made for giving quick results with expired air. Three of these are known as the alcometer, the intoximeter, and the drunkometer. Whatever you may think of these names, you could not fail to be impressed with the ingenuity of the machines. There is, however, some doubt about the value of the results they give. There is *generally little difficulty in obtaining*

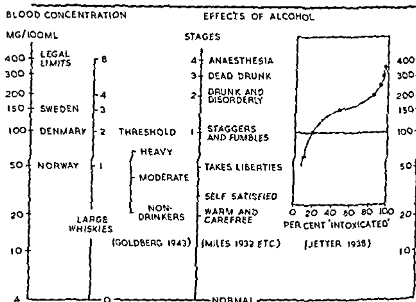


Fig. 1

a sample of urine and the B M.A. committee recommends that urine should normally be used for estimates of alcohol. The concentration is likely to be 33 per cent higher than the concentration in the blood during the time when that particular sample of urine was being formed by the kidney. The difficulty of knowing when this was, introduces an element of uncertainty, but this is not thought to make the test useless. Estimates on blood are probably the most satisfactory when it is possible to obtain a sample of blood collected with proper precautions at a known time, but this is not always easy.

Men and women who find themselves suspected of an offence against the law are sometimes reluctant to provide specimens

were six tests, in which real obstacles were replaced by light wooden posts and boards. The car was extricated from an artificial garage, driven along a curve, backed along a board, turned in a narrow 'road', driven into and out of sand and parked in a small space. When an obstacle was hit the test was repeated. The total time taken, including the repetitions, gave a measure of the skill of the driver. The effect of practice was eliminated by taking the differences between the times taken by alcoholic and control groups. The mean concentrations of alcohol in the blood were between 40 and 50 mg./100 ml. and this produced a significant loss of skill in driving as measured in this way. These results may be used to justify the Norwegian law, although it is probable that most drivers overcompensate for the effects of small amounts of alcohol by driving slowly with unusual care, and do not try to get the job done as quickly as possible as those who took part in these tests were asked to do.

It may be said that any fixed legal limit for the amount of alcohol which may be taken before driving a car is unfair on the man with a good head. On the other hand, it must be convenient in some ways to know just how much one can take, and a law which is so easy to administer must save the public much of the money that is spent in litigation in countries such as ours. It is logical to put driving with a high blood-alcohol in the same category as driving with defective brakes.

In Norway the penalties for alcoholic driving are severe and respected persons may easily find themselves in jail. The question of how heavy the penalty should be, must be distinguished from the question of what should constitute an offence. It may well be that the law of this country will eventually make it an offence to drive a car with a high blood-alcohol, but will not punish offenders so severely as is done in some other countries.

Chronic Alcoholism

The tolerance which may be acquired by the regular consumption of large amounts of alcohol has already been discussed. It is mild compared with the tolerance which may develop for morphine, and withdrawal of the alcohol does not normally produce symptoms analogous to those caused by the

to blame. They may prove that the pedestrian had taken alcohol; they may incriminate a corpse.

There is thus much to be said for the view that chemical estimates of alcohol should be more frequent than they are. The accused should certainly be entitled to insist on them; the police should probably also be entitled to insist on them, but a chemical estimate by itself cannot prove that a man was under the influence of drink and that is what the law of our land forbids. In some other countries it is illegal to drive a car after drinking more than a certain amount of alcohol. In Norway, for example, it is illegal to drive a car with more than 50 mg./100 ml. of alcohol in the blood. This means that you can have one large whisky (2 oz.) but no more. In Denmark you can have two large whiskies and in Sweden you can have three large whiskies and no more.

The fact th[illegible] authorities [illegible] that even in Norway alcohol may affect the driving of cars without exceeding the legal concentration in the blood. This concentration (0·05 per cent) does not produce clear clinical evidence of intoxication, except in a small percentage of people (Jetter, 1938), but it increases self-confidence in most people, and it seems probable that accidents are generally due to carelessness rather than to inco-ordination. The ordinary man is more likely to be careless on the way home from a party than when he is being tested by a scientist. Most of the tests which have been used have been tests of the efficiency of the brain, and drivers whose brains are reasonably efficient, but whose minds are not concentrated on the job, are more likely to have accidents than normal people. Most people agree that a carefree attitude to the world is produced by quite small amounts of alcohol and this fact may be used to support the argument that quite small amounts of alcohol should be forbidden.

This argument may be criticized as vague, but the experiments of Bjerver and Goldberg (1950) give more definite evidence in support of much the same conclusion. These two pharmacologists tested the effect of alcohol on the actual driving of cars. The drivers were mostly expert instructors and there

as an anthelminthic and discovered accidentally that, after they had taken it, alcohol produced disturbing after-effects. They showed that this was because disulfiram inhibits the oxidation of acetaldehyde which then accumulates in the body and causes flushing, headaches, nausea and vomiting. The patient is first given the drug followed by alcohol so that he may have first-hand knowledge of the effects. He then takes the drug at regular intervals and finds it easier to refuse drinks than it had been. It may be sufficient to take the drug twice a week, and some of the results obtained under supervision, first in Copenhagen and later elsewhere, have been very satisfactory.

In conclusion I must apologize for giving a superficial account of a great social problem. Alcohol leads to accidents and may cause madness, but it has done much to alleviate the sufferings of mankind and few of us would welcome the effective prohibition of it.

REFERENCES

Bjerver, K. and Goldberg, L. (1950). *Quart. J Stud Alc.* 11, 1
B M A. Committee (1954). *The recognition of intoxication.*
Falstaff, J. (1405). *Henry IV (2)* 4, 3.
Goldberg, L. [illegible]
Jacob [illegible]
Jettei [illegible]
Krae [illegible]
Warren, J. W. (1887). *J Physiol.* 8, 311

withdrawal of morphine. Occasionally it may be followed by delirium tremens, which is an acute psychosis accompanied by visual hallucinations which seem so real that, even afterwards, it may be difficult to believe that they were products of the imagination. Delirium tremens may also be produced by other factors which cause additional nervous stress in the drinker, such as bodily trauma, acute infections, or a specially heavy bout of drinking. When it follows attempts to withdraw alcohol it is probably due to emotion, rather than to a direct effect of a relative deficiency of alcohol. Death may occur from exhaustion or intercurrent infection; the patient should be quietened with sedatives, kept warm in bed, and treated according to his symptoms.

Heavy drinkers also often have *peripheral neuritis, cirrhosis* of the liver and fatty hearts and kidneys. There is some doubt whether these changes are directly due to alcohol or not. They may be due to the poor diet on which these people often live, and the administration of aneurine and other vitamins has been recommended. This form of treatment may well be beneficial, but the results have not been so striking as to prove that any one of the pathological changes observed is entirely due to deficiency of a vitamin. It must also be remembered that intoxicating drinks contain many other poisons besides alcohol. Alcohol itself can however probably be regarded as the main cause of the gastritis which follows the drinking of strong spirits.

The most important part of the treatment of chronic alcoholism is to teach the patient to avoid excessive alcohol. Once the habit of drinking has become ingrained it is difficult to eradicate it and still more difficult to prevent relapses. There is often an underlying mental disorder, but suitable psychological treatment may do real good. In America there are organizations known as the Yale School of Alcoholic Problems and Alcoholics Anonymous, which are devoted to helping drunkards. In many countries there are trained social workers who specialize in this kind of work, teaching their patients to avoid the places where people drink. Their work is sometimes aided by disulfiram (also known as antabuse), which makes alcohol seem less attractive. Jacobsen (1952) and others hoped originally to use this drug

body temperature falls progressively until death from respiratory and cardiac arrest.

Provided shivering is abolished the body temperature can be lowered in a variety of ways. The most obvious is by reducing

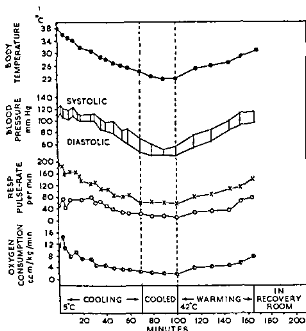


FIG 1. Mean results of cooling twenty dogs to 24–22° C

the ambient temperature of an anaesthetized or paralysed animal when the animal will lose heat through the skin. In practice immersion in cold water or envelopment in rubber blankets through which cold water circulates are the methods of choice. No risk of damage to the skin exists provided the surface temperature is not carried below 0° C. The simplicity of this method has much to recommend it though there is a real disadvantage in the difficulty of accurately controlling the level of hypothermia induced.

The most important alternative is that of direct cooling of the blood stream, either by the creation of an arterio-venous fistula (Boerema and others, 1951; Delorme, 1952) when the blood

XV

Cooling of the Whole Organism

D. G. MELROSE

Introduction

MAMMALS, and particularly man, enjoy a state of relative freedom from their environment through the *development of a complex mechanism of response*, designed to maintain in the body certain constants. This is the 'milieu interne' of Claude Bernard, the 'homeostasis' of Cannon. This concept, a keystone of physiology, has long guided our approach to the treatment of disease. Now a new principle is emerging which may have profound results.

In Britain hypotensive anaesthesia has become common *practice, and both here and in the United States of America* general hypothermia is being used increasingly by the cardiovascular surgeon. In France one hears of 'artificial hibernation' and of 'vegetative disconnection'. These techniques have a common factor, that of a deliberate temporary alteration of the 'milieu interne' in order to assist in the management of the patient. It is as yet too early to assess the importance of this trend, it is sufficient to recognize its intent.

It is surprising how little is known of the physiology of cold and of hypothermia in view of the fact that temperature is such a fundamental biological parameter. Cold and hypothermia must be clearly distinguished. Cold is a physical state—normally warm-blooded animals react to cold by vigorous attempts to maintain the body temperature. This involves muscular activity as shivering and associated increases in oxygen consumption and cardiac output. If this response is depressed in any way then a state of hypothermia ensues whereby the general

pressure respirator running at 24 cycles a minute at a pressure of plus 12 to minus 3 mm./Hg. All respiratory activity was paralysed with succinylcholine chloride. A closed-circuit carbon-dioxide absorption system was used and fresh supplies of oxygen were admitted through a one-way valve leading from a Benedict-Roth recording spirometer filled with 100 per cent oxygen. Measurements of O_2 consumption were obtained over 5 to 10 minute periods for every 2 degree drop in body temperature and continued after electrocardiographic evidence of death for an hour during which time a correction for any leaks in the system was obtained.

When the results obtained in this manner were plotted and compared with those obtained in the previous series striking differences were noted (Figure 2).

The average mean O_2 consumption at normal temperature in those dogs breathing spontaneously was 12·5 ccs./Kg./min. —in those on controlled respiration it was 9·0 ccs./Kg./min. The absence of respiratory movement and indeed of all somatic muscular activity is alone responsible for a reduction in O_2 consumption such as is obtained by a reduction of body temperature of 5° C. In this context it can also be seen that in the first series of dogs there occurred isolated oxygen consumption values comparable to the figure of 9 ccs./Kg./min. when the body temperature had dropped to 28° C. Thus it is evident that hypothermia if it is to be successful in reducing the basal metabolic rate must be induced without stress and without shivering.

Another important difference is revealed when attention is directed to the temperature to which those dogs on controlled respiration were taken. All the animals in this group survived a body temperature of 20° C., and at 16° C. eight were still alive. Five more were alive at body temperatures between 14·5 and 13 5 C. and one dog did not die until its body temperature had reached 11° C

The mechanism whereby controlled respiration acts is obscure and the only definite finding is that this method maintains the blood pressure at a much higher level throughout the period of cooling. At a body temperature of 22° C. when 15 per cent of

circulates from artery to vein through a length of plastic tubing immersed in coolant or by driving blood along such a tube from vein to vein (Ross, 1954). This latter method requires a pump. Advantages claimed are that the rate of cooling is augmented and that more control of the temperature-drop can be obtained. To set against these are the obvious disadvantages accompanying extracorporeal circulation of blood. Other methods suggested have been cooling of the lungs directly by intratracheal insufflation of cold air or by intrapleural lavage, and work has also been done on intraperitoneal lavage. No real physiological distinction need be made between these various methods. Each has its particular advantages and disadvantages and the choice is dictated more by the circumstances for which cooling is required rather than by physiological principle.

As the body temperature falls the metabolic activity declines and there occur associated haemodynamic changes.

The mean values of the body temperature, blood pressure, pulse rate, respiratory rate, and oxygen consumption of 20 dogs cooled to 24–22° C. are shown plotted against time in Figure 1. Each of these values falls progressively as the body temperature is lowered and rises again as the body temperature is restored to normal. In this study it was noted that the O_2 consumption when measured at temperatures above 28° C. depended largely on the type and depth of anaesthesia and that it showed considerable variations from dog to dog and even in the same dog: below 28° C. a strikingly uniform reduction was apparent. It was found too that the temperature at which natural respiration ceased varied widely and was also dependent on the depth of anaesthesia and particularly on the amount of barbiturate used.

Controlled Respiration

In order to circumvent these anaesthetic effects a further series of studies were done in which the respiratory exchange was accurately controlled throughout the period of cooling. This was done by connecting the animals, after intubation of the trachea with a cuffed tube, to an automatic positive-negative

evidence has been presented for this. The beneficial effects may be due to the ability of the method to maintain the coronary circulation at a higher level—certainly it has been reported that controlled positive-negative respiration will maintain the

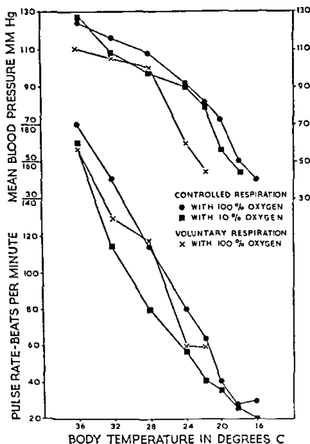


Fig. 3 The reduction of pulse rate and blood pressure during hypothermia.

cardiac output (Brecher, 1953). Other authors have commented on the beneficial effects of controlled respiration and regard it as essential (Bigelow and others, 1950; Swan and others, 1953).

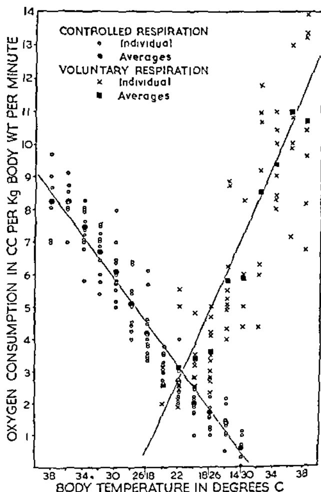


FIG 2 —
values o
on the
indicate averages

dogs not on controlled respiration were dead but all of those being artificially respired were alive, the blood pressure in the latter group was approximately twice that of the former (Figure 3).

It may be that acapnia attending controlled respiration with carbon dioxide absorption is the responsible factor but no clear

evidence has been presented for this. The beneficial effects may be due to the ability of the method to maintain the coronary circulation at a higher level—certainly it has been reported that controlled positive-negative respiration will maintain the

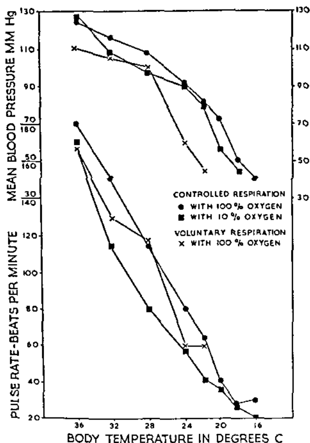


FIG 3. The reduction of pulse rate and blood pressure during hypothermia

cardiac output (Brecher, 1953). Other authors have commented on the beneficial effects of controlled respiration and regard it as essential (Bigelow and others, 1950; Swan and others, 1953).

Cardiac Arrhythmias

The most serious problem encountered in the reduction of the body temperature is the incidence of cardiac arrhythmias leading frequently to spontaneous ventricular fibrillation or to cardiac arrest. Below 28° C. in man and in the dog the likelihood of its occurrence increases progressively when the temperature is reduced and below 20° C. it may be uniformly expected. The reasons at present are unknown and no method yet advocated has consistently produced permanent restoration of normal cardiac rhythm below 22° C. In our hands attempts to reduce the onset of these irregularities were not generally successful.

Three main approaches were explored. The first of these was hypoxia of the myocardium. Anoxic anoxia was immediately excluded for full arterial saturation was maintained. Also it was shown that coronary sinus blood was normally desaturated suggesting adequate tissue uptake. However, Bing and others (1949) pointed out that the myocardium extracts considerably more oxygen from blood than do other tissues, and they showed that increased demand is met by an increased perfusion rather than by further arterial desaturation. Also Lange and others (1949) reported that hypothermic rabbits responded to a greatly increased atmospheric pressure of oxygen by a restoration of the electrocardiogram to normal. Accordingly with the kind help of the Royal Naval Physiological Laboratory a series of dogs were cooled and exposed to oxygen at 70 lb./sq. in. Unfortunately no clear evidence has been obtained from these experiments but certainly no solution of this problem is provided by this manoeuvre.

A series of animals were cooled in the usual manner to about 25° C. and then placed in a pressure chamber. Cooling was continued until evidence of cardiac arrhythmia was seen on the lead II electrocardiogram whereupon oxygen was pumped into the chamber until the pressure had risen to 70 lb./sq. in. This pressure was maintained for periods up to one hour and then released. Tracings obtained from the electrocardiogram showed some improvement in 40 per cent of the animals, no change in 20 per cent and deterioration in 40 per cent.

Attention was then turned to the chemical environment of the heart. Plasma sodium, potassium, calcium and magnesium were measured together with plasma chloride and bicarbonate.

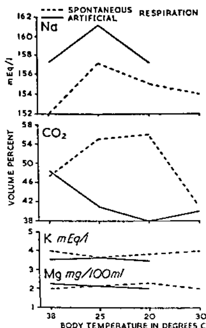
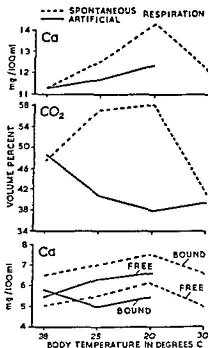


FIG. 4 The alterations in sodium, potassium and magnesium during reduction of body temperature to 20° C and rewarming to 30° C Note that the electrolyte changes are the same whether the carbon dioxide content of the blood rises or falls

Potassium and sodium together with bicarbonate and chloride altered in a manner consistent with the known changes occurring in respiratory alkalosis and acidosis. Differences between those animals breathing spontaneously and those on controlled respiration emphasize this (Figure 4).

The plasma calcium, however, rose in both groups (Figure 5). The most pronounced rise occurred below 25° C No reliable figures for the amount of ionized calcium present could be obtained but it is likely that this value was higher in the group showing respiratory acidaemia That calcium is implicated as

a potentiator of fibrillation is supported by additional evidence. The effect of $CaCl_2$ infusion on the electrocardiogram of dogs at normal body temperature was compared with the effect of the same infusion at a reduced temperature. There is strong evidence that the 'current of injury' potential in association with the 'S' deflection occurring at low body temperatures described by Osborn (1953) is due to the presence of an



increased amount of ionized calcium. It would be strange if an alteration in the concentration of an ion of such importance to an orderly heart beat did not have an important effect at low body temperature. This work is proceeding and may find confirmation.

The finding by Brown (1954) that acetylcholine secretion in the synapse is depressed at low temperature points to a third

possibility—that is that the conducting tissue in the heart is directly responsive to the cold and that as the grip of the pacemaker weakens the tendency to the formation of multiple ectopic foci of contraction leads to fibrillation. Some support for this is implicit in the finding by Prevedel, Montgomery and Swan (1954) that anticholinesterase significantly raises the fibrillating threshold. The absence of a really definitive method of inducing fibrillation makes the study of the problem very difficult and yet its solution will open the way to a much greater application of hypothermia.

In the temperature range above 20° C. major organ function continues, though at a very slow rate, and no specific cellular pathology occurs within periods of some hours. The temperature range below 20° C. has been much less explored and here I must mention the remarkable achievements of Smith, Lovelock and Parkes (1954). They have reduced the body temperature of rats and mice to zero ° C. and that of hamsters to a temperature low enough to produce ice crystallization in the skin. A proportion of these animals recovered completely. It may be that the rodent species which include the hibernating animals have a special defence against cold injury but there is good evidence to suggest that this is not the case. Great interest attends the development of this work and its extension to larger mammals.

Pharmacological Agents

In France a rather different technique exists based on the use of pharmacological agents combined with a mild degree of cooling (Laborit, 1952). Extensive claims have been made for this method in the treatment and prevention of shock though as yet no conclusive evidence is presented. Chlorpromazine and promethazine—two of the agents employed—have found favour in this country as adjuncts to anaesthesia having very useful properties in premedication. The claims that they specifically reduce body temperature and metabolism have not been substantiated by Shackman and others (1954).

Comparison between the oxygen consumption of a group of patients before and after receiving chlorpromazine did not

reveal any significant reduction in the basal metabolic rate. To contend that a combination of these drugs alone produces a state comparable to hibernation or hypothermia is irrational for this study revealed that as well as producing scant effect on the oxygen consumption, it usually produces no change in body temperatures in a normally covered patient. However, chlorpromazine does produce peripheral vasodilatation with an increased peripheral blood-flow and inhibits in part the tendency to shiver. For these reasons it is a useful adjunct in the induction of hypothermia.

Possible Clinical Application

The primary stimulus to the study of hypothermia has come from the clinical field, though there have been striking examples outside this. Francis Bacon is said to have met his death from pneumonia while refrigerating chickens with intraperitoneal snow. John Hunter was fascinated by hibernation and requested Jenner to investigate the digestion of hibernating hedgehogs. Troedsson in 1939 pointed out the surgical possibilities of hypothermia on the results of his experiments on rabbits, and Woodruff (1941) showed that their findings were applicable to the dog. But the work of Smith and Fay (1939, 1940) and Talbott (1941) was directed to clinical problems, that of the management of advanced cancer and of severe mental disturbance, and the credit for our present-day interest must go to Bigelow in Toronto (1950) for his suggestion and experimental proof that the period during which an animal would withstand circulatory arrest could be greatly prolonged by hypothermia. Out of his work and that of Boerema arose a wealth of clinical applications and the reawakening of biological interest.

There is no doubt that general hypothermia is capable of significantly reducing metabolism and of producing hypotension. As such it may be considered potentially valuable in a variety of conditions.

It may find a place in the treatment of reversible conditions causing hypoxia. Acute pulmonary disease such as pneumonia and atelectasis, anaemic crises and cyanotic heart disease are

examples. In this category also come instances of deliberate temporary interruptions of blood supply whether it be total interruption in order to provide a dry intracardiac field or regional interruption to enable aneurysmal dilatations to be corrected.

The fall in blood pressure accompanying hypothermia may aid the plastic surgeon in massive skin grafting, the neurosurgeon in cranial resection and perhaps the physician in his treatment of internal haemorrhage from oesophageal varices.

The specific effect of lowering the temperature may be useful in the management of thyrotoxicosis, heat stroke and severe infection.

At the present time these instances must be regarded as conjectural. It is only in the field of cardiovascular surgery that encouraging experimental and clinical experience is accumulating. McQuiston (1949) reported the protective effect of mild hypothermia in the operative management of cyanotic heart disease; Lewis and Taufic (1953) and Swan and Zeavin (1954) have reported various successful intracardiac operations under direct vision. The operative management of great vessel resection and grafting is greatly simplified by the protective action of hypothermia and it is perhaps in this particular field that it has its greatest use and clearest justification. Experience confirms too that the younger the animal or patient the greater the margin of safety and in the very young mammal much lower temperatures can be obtained without danger (Adolph, 1951). Infants whose lives were endangered by the severity of congenital cardiac deformities have thrived when nursed in an environment controlled to provide continuously a body temperature of 30° C. (Juvenelle, 1954).

These few suggestions may be hopelessly optimistic but they serve to reveal the scope of this method However, in spite of the great promise that hypothermia may hold it must be considered as a procedure involving serious inherent risks While not denying the right of individuals to a more immediately optimistic view it is proper that until further knowledge lends safety, experience should be gained in the experimental labor-

atory where legitimate controls may be established, objective data obtained and the results tested statistically.

ACKNOWLEDGEMENTS

Much of the data presented in this lecture was obtained during research into problems of hypothermia at the Buxton Browne Research Farm in Kent, by McMillan, Melrose, Churchill-Davidson and Lynn (1955). For permission to include previously published material I am indebted to the Editors of the *Lancet* and the *Annals of the Royal College of Surgeons*.

REFERENCES

ADOLPH, E F. (1951). *Amer. J. Physiol.* 166, 75.
BIGELOW, W G., LINDSAY, W. K. and GREENWOOD, W. F. (1950). *Ann Surg* 132, 849.
[illegible] SPENCER,
[illegible] J. H. and
[illegible]
[illegible] UYSEN, L. (1951). *Arch. Chir. neerl.* 3, 25
[illegible] 74, 299.
[illegible]
[illegible] 73.
[illegible] Paris.
[illegible] *n. Med* 31, 989.
[illegible]
[illegible] C. and
[illegible]
[illegible]
[illegible] (1954) *Proc. Soc. exp. Biol., N.Y.* 85, 596.
ROSS, D. N. (1954) *Lancet*, i, 1108.
SHACKMAN, R, WOOD-SMITH, F G, GRABER, I G., MELROSE, D. G. and LYNN, R. B (1954) *Lancet*, ii, 617.
SMITH, L W and FAY, T. (1939) *J Amer med. Ass.* 113, 653.
SMITH, L. W. and FAY, T. (1940). *Amer. J. clin. Path* 10, 1.

SMITH, A. U., LOVELOCK, J. E. and PARKES, A. S. (1954). *Nature, Lond.* **173**, 1136.
SWAN, H., ZEAVIN, I., HOLMES, J. H. and MONTGOMERY, V. (1953). *Ann. Surg.* **138**, 360.
SWAN, H. and ZEAVIN, I. (1954). *Ann. Surg.* **139**, 385.
TALBOTT, J. H. (1941). *New Engl. J. Med.* **224**, 281.
TROEDSSON, B S. (1939). *Arch. phys. Therapy*, **20**, 501.
WOODRUFF, L M. (1941). *Anesthesiology*, **2**, 410.

atory where legitimate controls may be established, objective data obtained and the results tested statistically.

ACKNOWLEDGEMENTS

Much of the data presented in this lecture was obtained during research into problems of hypothermia at the Buxton Browne Research Farm in Kent, by McMillan, Melrose, Churchill-Davidson and Lynn (1955). For permission to include previously published material I am indebted to the Editors of the *Lancet* and the *Annals of the Royal College of Surgeons*.

REFERENCES

ADOLPH, E. F. (1951). *Amer. J. Physiol.* 166, 75.
BIGELOW, W. G., LINDSAY, W. K. and GREENWOOD, W. F. (1950). *Ann. Surg.* 132, 849
BING, R. J., HAMMOND, M. M., HANDELSMAN, J. C., POWERS, S. R., SPENCER, F. C., ECKENHOFF, J. E., GOODALE, W. T., HAFKENSCHIEL, J. H. and KETY, S. S. (1949). *Amer. Heart J.* 38, 1
BOEREMA, I., WILDSCHUT, A., SCHMIDT, W. J. H. and BROEKHUYSEN, L. [illegible]
[illegible] 74, 299
[illegible]
[illegible] 31, 989.
LEWIS, F J. and TAUFIC, M (1953) *Surgery*, 33, 52.
MCMILLAN, I. K R., MELROSE, D. G., CHURCHILL-DAVIDSON, H. C. and LYNN, R. B (1955). *Ann r Coll. Surg , Engl.* 16, 186.
MCQUISTON, W. O. (1949) *Anaesthesiology*, 10, 590.
OSBORN, J J (1953). *Amer J. Physiol* 175, 389.
PREVEDEL, A. E., MONTGOMERY, V and SWAN, H. (1954). *Proc. Soc exp Biol., N.Y.* 85, 596
ROSS, D. N. (1954). *Lancet*, i, 1108.
SHACKMAN, R., WOOD-SMITH, F. G , GRABER, I. G , MELROSE, D. G and LYNN, R B. (1954). *Lancet*, ii, 617.
SMITH, L. W. and FAY, T (1939) *J. Amer. med Ass.* 113, 653.
SMITH, L. W. and FAY, T. (1940). *Amer. J. clin. Path.* 10, 1.

SMITH, A. U., LOVELOCK, J. E. and PARKES, A. S. (1954), *Nature, Lond.* **173**, 1136.
SWAN, H., ZEAVIN, I., HOLMES, J. H. and MONTGOMERY, V. (1953). *Ann. Surg.* **138**, 360.
SWAN, H. and ZEAVIN, I. (1954). *Ann. Surg.* **139**, 385.
TALBOTT, J. H. (1941). *New. Engl. J. Med.* **224**, 281.
TROEDSSON, B. S. (1939). *Arch. phys. Therapy*, **20**, 501.
WOODRUFF, L. M. (1941). *Anesthesiology*, **2**, 410.

XVI

Substances that Increase Tissue Permeability and their Relation to Infection and Fertilization

DOUGLAS McCLEAN

AN HISTORICAL approach will, I think, show best how interest in these substances developed and the gradual realization of their widespread distribution in biological material. This approach will also reveal an excellent example of the way in which a single crucial observation can bring together apparently completely unrelated work in entirely separate fields in different countries. In a general discussion covering a wide field it will be impossible to go into details of chemical structure or into the complicated question of subtle differences in the kinetics of the activity of related enzymes from different sources—questions which are still the subject of active investigation and debate. All I can hope to do is to stimulate interest in the part these substances may play in infection and fertilization and to indicate the scope for further work in both these fields. This should not only increase our understanding of some of the problems of infection and of infertility and excessive fertility; it should also throw further much needed light on the synthesis and metabolism of an important group of polysaccharides in the connective tissues. Although there are certain synthetic compounds which increase tissue permeability I shall confine myself to those of biological origin that were originally known as 'Diffusing factors'.

Early Work on 'Diffusing Factors'

Testicular Extracts

Duran-Reynals (1928, 1929; Duran-Reynals and Suner Pi, 1928) described the influence of fresh aqueous extracts of rabbit testicle on the infecting power of vaccinia virus. He reported that these extracts caused a marked enhancement of the lesions in the skin due to the virus; he concluded that the extract acted on the cells of the host rather than on the virus and he tentatively suggested that the effect might be due to increased cell division. Being interested at the time in the propagation of vaccinia both in tissue culture and in the skin we investigated the activity of these extracts. In addition to the increased infectivity of the virus another immediate activity of the testis extracts was obvious; instead of the usual well-marked and persistent bleb following intracutaneous injection, testis extract immediately diffused outwards into the dermis so that after thirty seconds it was difficult to identify the site of injection; the rapid diffusion was so striking that it could be compared to the different behaviour of a drop of water when placed upon glazed and upon blotting paper. This spreading action was the explanation of the enhancement of the virus lesion and was independently confirmed by Hoffman and Duran-Reynals (1931).

Further investigation (McClean, 1930 and 1931) showed *inter alia* that, although testis extract increased the size of vaccinial lesions and the yield of virus obtained therefrom, it did not decrease the minimal infecting dose of the virus. The enhancing activity of the extract could, however, be demonstrated in a dilution of 1 100,000 which indicated that we were dealing with a substance of great activity. The spreading effect which was also produced by aqueous extracts of spermatozoa *could be shown in skin up to forty-eight hours after its* removal from the killed animal and in skin which after removal had been desiccated and then soaked in water. The enhancing action was not confined to vaccinia virus; lesions following intracutaneous injection of staphylococci or of diphtheria toxin were increased in proportion to the amount of immediate diffusion produced by the extract.

Bacterial Diffusing Factors

In 1933 Duran-Reynals reported that aqueous extracts of invasive strains of staphylococci and streptococci contain a soluble factor that spreads like testis extract, enhances the infection produced by non-invasive strains of these organisms, by other bacteria and by vaccinia virus. Non-invasive strains do not contain this factor. Duran-Reynals defined invasiveness as the power of the injected bacteria to spread locally in the tissues and he distinguished this property from virulence, defined as the power to kill. As he said, although these two characters are usually combined there are strains which are virulent but not locally invasive and *vice versa*. Duran-Reynals also reported that diffusing factor elaborated locally by the organism passes into the blood stream and causes a general increase of tissue permeability.

During routine intracutaneous tests on horses with the toxins of *C. diphtheriae* and *Cl. welchii* we noticed that, whereas the injection bleb and the lesion following diphtheria toxin remained circumscribed to the area of injection, the bleb produced by welchii toxin disappeared immediately and the resulting lesion tracked down in the direction of gravity for as much as 35 cms. An investigation of the diffusing factors produced by *Cl. welchii* and other members of the gas-gangrene group (McClean, 1936) revealed that diffusing activity could be detected in as little as 0·000015 ml. of a toxin that had a minimum lethal dose for the mouse of 0·09 ml. The diffusing factors could be separated from the toxins produced by these organisms and, unlike the toxins, they were not inactivated by formaldehyde.

A survey of the production of diffusing factors by the commoner clostridia revealed that culture-filtrates of most strains of *Cl welchii* and *Cl. septicum* produce potent spreading factors though there is considerable variation between different strains of the same type of organism. Only a small proportion of strains of *Cl. oedematiens*, *Cl. welchii* (Type C) (*paludis*) and Type D (*ovitoxicus*) produce this factor and never to a high titre. No spreading was ever detected with filtrates of *Cl. tetani*. At the time we suggested that spreading activity was broadly correlated

with the local invasiveness and fulminating infectivity of the different species of this group. It may be mentioned here that many strains of pneumococci of different types were found to produce spreading factors and that some active strains rendered avirulent by repeated culture in specific antisera ceased to produce these factors. It was not possible, however, to correlate diffusing activity with virulence in clinical infections (Humphrey, 1944).

It may be useful to summarize the position at this stage. Substances which promoted spread in the dermis had been obtained from mammalian testes and spermatozoa and from a range of culture-filtrates from bacteria broadly characterized by their invasive qualities. They had also been obtained in significant concentrations in certain mammalian tumour extracts (Duran-Reynals and Stewart, 1931; Boyland and McClean, 1935), in several different species of snake and spider venoms (Duran-Reynals, 1939) and in leech extracts (Claude, 1937).

Considerable purification of both the testicular and bacterial factors had been achieved by relatively simple methods and as little as 10^{-5} mg. of dried preparations were active. We were still ignorant of the mechanism of this activity; experiments of my own with possible substrates such as collagen, gelatine and echinoderm ova had been completely negative. The physiological significance of the spreading factors in the spermatozoa was unknown.

The Hyaluronidases

In 1939 the whole question received a fresh impetus from apparently unrelated fields. Meyer and his associates (1940a, b), described a bacterial enzyme derived from pneumococci of more than one serological type which hydrolysed a mucopolysaccharide in synovial fluid. This polysaccharide is also present in the vitreous humour of the eye, in Wharton's jelly of the umbilical cord and in skin and muscle as a cement substance. During the investigation of a sample of synovial fluid accidentally contaminated with *Cl. welchii*, Robertson, Ropes and Bauer (1940) discovered an enzyme produced by this organism that hydrolysed and reduced the viscosity of synovial

fluid. Meyer and Palmer (1936) had called the polysaccharide substrate hyaluronic acid and hence the enzymes acting on it were called hyaluronidases. Meyer described the polysaccharide as being a long chain molecule composed of equi-molar parts of glucuronic acid and *N* acetyl glucosamine. It is interesting to note that all the sources of the polysaccharide are mesodermal in origin.

At this time nobody suspected any connection between hyaluronidases and diffusing factors. It remained for Chain and Duthie (1939, 1940) to identify the two. They reported that purified preparations of testicular diffusing factor showed a remarkable mucolytic activity which caused a rapid fall in the viscosity of mucoprotein preparations with the liberation of reducing substances. They suggested that the mucolytic and diffusing activities might be due to the same factor and that the spread in the tissues might be due to the action of the enzyme on the mucin-like interfibrillar substance in the collagen of the dermis. This suggestion was further substantiated when they isolated from the skin a substance indistinguishable from hyaluronic acid, the viscosity of which was reduced by diffusing factor. They found that spreading factors from sources other than the testicle had a similar mucolytic activity.

The identity of these mucolytic enzymes—or hyaluronidases—and diffusing factors was rapidly confirmed by workers both in this country and in America. The activity of the enzymes were studied and different methods of titration were devised and compared with each other and with the measurement of activity in the tissues (Madinaveitia *et al.*, 1940; Madinaveitia and Quibell, 1940; Meyer *et al.*, 1940a, b; Hale, 1944a, b; McClean and Hale, 1941; McClean, 1943). The more important methods of measuring enzyme activity are:

1. Diffusion in the skin. Not less than 20 per cent increase in area of spread of haemoglobin solution or other indicator (Humphrey, 1943). This is probably still the most delicate method of detecting the presence of hyaluronidase but the least accurate for estimating activity.

2. Viscosimetry (McClean and Hale, 1941). Reduction in the viscosity of solutions of hyaluronate This is not a delicate

method of detecting activity but, as a method of titration, it is probably accurate to about ± 10 per cent.

3. Liberation of reducing sugars from hyaluronate solutions. The reaction is slow and is not a delicate method of titration.

4. Mucin clot prevention test (McClean, 1943). This method depends upon the destruction by the enzyme of the power of a hyaluronate-serum mixture to clot in the presence of acetic acid. The activity estimated by this test is roughly similar to that detected by diffusion in the skin and it is accurate to about ± 20 per cent.

5. Turbidimetry. This method depends upon the reduction by the enxyme of the turbidity of mixtures of hyaluronate and acid serum. The principle is similar to that of the M.C.P. test and it has been extensively used in recent years; it was introduced by Seastone (1939) and later modified by Pike (1946) and Humphrey and Jaques (1953).

It was thought that these methods might be measuring different enzymic activities and later work by Rogers (1945, 1946a, b, 1948), Hahn (1945a, b, 1946a, b) and others has shown this to be true. The mode of action of these enzymes differs in detail according to their source. This is, however, a complex question that I cannot pursue here; I can only say that the breakdown of the polysaccharide occurs in a series of stages and that the final products can differ considerably according to the source of enzyme and the conditions of hydrolysis

Immunity to Hyaluronidase

McClean (1936) had produced antisera which neutralized the skin-diffusing activity of the culture filtrates, and it was later shown that these sera would also neutralize the viscosity-reducing and mucin clot preventing activities of the contained hyaluronidases (McClean and Hale, 1941; McClean, 1943). These sera could be prepared free from antitoxin and the anti-hyaluronidase activity of available antitoxins is independent of the antitoxic potency. These antisera were shown to be species specific for the clostridial enzymes, group specific for streptococci and species specific for the testicular enzymes.

Relation of Bacterial Hyaluronidases to Infection

When *Cl. welchii* is grown in a medium containing hyaluronate a hundredfold increase in the yield of hyaluronidase results (Table 1) (McClean and Hale, 1941).

TABLE 1. *The Influence of Hyaluronate in the Culture-Medium of Cl. welchii*

Medium	Bacterial Multiplication opacity	Hyaluronidase v.r.u.	Highest dilution diffusing in skin
Peptone broth alone	1	13	10^{-5}
Peptone+0·5% glucose	8	70	10^{-7}
Peptone+0 5% hyaluronate	8	1340	10^{-9}
Peptone+0 5% hyaluronate+ 1 unit testis hyaluronidase	8	1660	10^{-9}

The increase in the yield of enzyme with the addition of glucose alone is probably due to the increase in bacterial multiplication; the much greater response to hyaluronate is not accompanied by any greater growth. The addition of a small amount of hyaluronidase to initiate the breakdown of the hyaluronate causes no further significant increase in growth or yield of enzyme.

Since spreading and viscosity-reducing activities are both increased by the addition of hyaluronate this experiment provided confirmatory evidence that diffusion in the tissues is due to hyaluronidase; it also indicated that the enzyme is produced adaptively by the organisms. This was subsequently shown by Rogers (1945) to be true for all other hyaluronidase-producing organisms except staphylococci and *Cl. septicum* in which the enzyme is constitutive and does not respond to hyaluronate in the medium. Rogers also showed that the response of streptococci and *Cl. welchii* is directly proportional to the amount of



FIG. 1a. A collection of several ova surrounded by the cumulus cell mass and the corona radiata.



FIG. 1b A similar group of ova immediately after treatment with hyaluronidase, the eggs are floating freely and the cells in the background have fallen to the bottom of the watch-glass

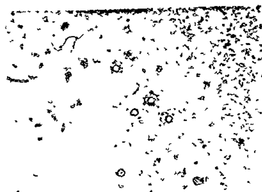


FIG 1c A few ova in which the gel is almost completely dissolved but in which its outline and a few remaining adherent cells can be seen

hyaluronate added to the medium. The discovery of this adaptive response also has further implications for the part that these enzymes may play in infection: the diffusing activity of hyaluronidase in mesodermal tissues not only spreads the organisms more widely, it also renders more of the polysaccharide available as a metabolite for them and this in turn increases the production of the enzyme; thus a vicious circle is established which promotes the extension of the infection. It remains to show that the course of infection and the toxicity, virulence or invasiveness of the organisms is in fact influenced by the presence of these enzymes.

Before turning to the special case of streptococci, I should like to refer briefly to the results of some experimental infections with clostridia carried out during the war in the hope of improving
1943).
of anir
with those that do not produce this enzyme: with the former strains there was widespread non-viscous oedema fluid soon after the infection was established; with the other strains the oedema fluid was relatively localized and highly viscous The viscosity could be shown to be due to hyaluronic acid since it was immediately destroyed by the addition of hyaluronidase from any source. Mixed infections, in which hyaluronidase-producing streptococci or staphylococci, relatively harmless alone, were added to non-hyaluronidase-producing clostridia, yielded some dramatic results in accelerating the spread of infection and the early death of the animals. The synergic action of mixed infections was particularly marked when the hyaluronidase-producing organisms were added to those with predominantly proteolytic activity such as *Cl. histolyticum.*

Both the bacterial hyaluronidases and the specific toxins could be detected in the oedema fluid very early in the experi-
seful in
nfirmed
, 1944;
McClean and Rogers, 1944) suggested that this failure was probably due to the large prophylactic doses of antitoxin which

also contained anti-hyaluronidase given as a routine to the wounded.

Before leaving the question of mixed bacterial infection I should like to refer to an interesting piece of work by Lack (1948) illustrating the influence of hyaluronidase on viral lesions in mixed infections. He reported that both staphylococci and streptococci, which produced minimal lesions alone, when inoculated intracutáneously together with vaccinia virus caused a markedly increased viral lesion and were themselves spread over a large area. The enhancing activity was proportional to the hyaluronidase produced by the cocci. This is another example of the synergism in mixed infections which was seen with clostridia and Lack suggested that it might be an explanation of the fulminating staphylococcal and streptococcal infections known to occur with smallpox.

The Streptococci

The streptococci present a special case because of the relationship between hyaluronidase produced by these organisms and the capsular material. Consideration of their significance entails another excursion into history.

Fifty-eight years ago, Bordet (1897) pointed out that virulent streptococci develop capsules in the infected animal and resist phagocytosis whereas those organisms that fail to develop capsules are destroyed. Cowan (1922) reported that the virulence of streptococci is associated with strains that produce a 'Mucoid' type of colony on solid media. In 1931 Hare showed how the resistance of the organisms to phagocytosis diminished as the cultures aged and Seastone (1934) correlated this decreased resistance with the loss of capsule in the older cultures. Meanwhile, Lancefield (1928) had isolated the M substance in group A streptococci which is responsible for their type specific reactions; the antibody to this substance appears to play a major part in protection from infection with this group. Kendall, Heidelberger and Dawson (1937) isolated the capsular substance of group A strains of streptococci and proved its identity with the polymerized hyaluronic acid isolated by Meyer and Palmer (1934), from vitreous humour and later

shown to occur in the capsules of group C streptococci, the skin, synovial fluid and other mesodermal tissues. Thus the virulence of streptococci has been shown to be associated in some degree with their capsules though probably in Group A strains Lancefield's M antigen is more important; in Group C strains it appears that the capsule is the important factor in virulence.

The capsules of both Group A and Group C streptococci are composed of hyaluronic acid (McClean, 1941a, b) and we now know that this is a normal body constituent of animal tissue which is not antigenic and will not act as a hapten (Humphrey, 1943). Some strains in each group produce capsules, others produce hyaluronidase. Hyaluronidase from any source destroys the capsules; the process can be watched and is like the melting of a lump of sugar in hot tea. A visitor to our laboratory from America who was shown this phenomenon remarked, 'I guess this enzyme sure strips the pants off the streptocoxi'. This seemed to us to be an apt comment. Hyaluronic acid thus seems to be essential to the integrity of the capsule. Hyaluronidase production, on the other hand, is stimulated by the inclusion of hyaluronate in the medium, as in the clostridia.

At this time it seemed as though enzyme and capsule production were mutually exclusive. It was suggested that normal capsule loss in ageing cultures might be due to traces of hyaluronidase but no evidence for this was found. Morrison (1941) had, however, reported that ageing cultures produce a thermolabile substance that would destroy capsules. Pike (1948) confirmed that ageing cultures of some but not all capsulated Group A streptococci contain a labile filterable substance that might be a hyaluronidase. Recently MacLennan (1956a and b) has established, *inter alia*, that serum is not necessary in the medium for capsule production if enough glucose is present, that some, but not all, capsulated Group A and Group C strains produce an enzyme which he has characterized as a hyaluronidase and that the enzyme produced by these strains is extremely thermolabile, is protected by the presence of its substrate and is serologically distinct from the enzyme produced by uncapsulated strains of the same group. MacLennan also pointed out that the

variable enzyme activity in different capsulated strains explains the lack of correlation between capsulation and the amount of hyaluronic acid present in the culture. In some strains the presence of capsules and of hyaluronic acid in the medium represents the excess of synthesis over enzymic destruction. He was unable to demonstrate any hyaluronidase-producing mutants in capsulated strains. Faber and Rosendal (1954) have recently reported a survey of 78 strains of group A haemolytic streptococci representing 40 different serological types for hyaluronidase and hyaluronic-acid production. *Both functions* [illegible] uronic-acid production is not a function of certain serological types but of individual strains.

The distinction already referred to, drawn by Duran-Reynals (1933) between virulence and local invasiveness must be borne in mind when considering the parts played by capsules and by hyaluronidase in infection. Broadly speaking, hyaluronidase-producing strains have a low virulence for such animals as mice when they are introduced by the peritoneal route, but they cause spreading local lesions as in the skin of rabbits and guinea-pigs; the enzyme enhances the local action of the toxins. Capsule production, on the other hand, is related to virulence but the presence of the M antigen seems to be more important in the Group A strains. Capsules are more important in determining the virulence of Group C strains which more commonly cause infection in animals. Rothbard (1948) has, however, shown that even in Group A infections there is a synergistic action of the M substance and the capsule. Thus although hyaluronidase alone protected against only 10 mouse M.L.D. and anti-M sera protected against 100,000 M.L.D., a combination of the same quantities of both the enzyme and antibody protected against a million M.L.D.

How does the capsule contribute to the virulence of streptococci? It is a viscous envelope which, being non-antigenic, presumably does not stimulate the production of antibodies which can sensitize the cocci to phagocytosis or the bactericidal action

of complement. It forms a gel inside which the organisms appear to multiply. This is strikingly apparent after the intraperitoneal injection of mice: with a capsulated strain a scanty viscous secretion packed with streptococci is found, whereas a hyaluronidase-producing strain provokes a copious fluid exudate. It was hoped that infection by capsulated strains might be controlled by repeated intravenous injections of hyaluronidase. Attempts to achieve this (Hirst, 1941; McClean, 1941; Kass and Seastone, 1944) produced conflicting results; the Americans obtained slight but definite protection whereas I failed completely. Since the dosage of enzyme given by me should have been adequate I think my failure was due to the use of a highly purified enzyme which was subsequently (McClean, 1942) shown to be very rapidly inhibited in the body, apparently by competitive adsorption.

Many American authors have reported the presence of specific anti-hyaluronidase in the sera of patients suffering or recovered from infections with hyaluronidase-producing organisms. This is, however, of limited significance in assessing the importance of the enzyme in infection; the body will produce antibodies to any antigen that is presented to it, regardless of the antigen's importance in any invasive process.

In conclusion, it can be said that it is doubtful whether the enzyme alone can determine the nature of the infection but highly probable that it influences the spread of the organisms and of other bacterial products such as toxins. It also increases the supply of a useful metabolite present in the tissues—hyaluronic acid. This substrate can not only be used by the organisms as a metabolite; as a capsule it can influence their virulence and survival. I would like to plead for more work to elucidate the role of enzyme and substrate especially in mixed infections. This work should also throw additional light on the metabolism and synthesis of these important mesodermal polysaccharides.

FERTILIZATION

Rowlands (1942) reported that ovulation in the rat could be produced by an injection of mare's serum (the follicle stimulating hormone) followed by chorionic gonadotrophin. The

eggs were collected from the Fallopian tubes and were surrounded by a zona radiata and cumulus cell mass which Rowlands stated was dispersed by the supernatant fluid from a sperm suspension. This observation suggested the possibility that hyaluronidase might be responsible for the dispersion of the cell mass. Previous attempts to elucidate the part played by hyaluronidase in fertilization involving experiments with echinoderm ova, bovine cervical mucin and the mucin from the ape's sexual skin had been uniformly negative. The presence of a transparent gel in which the cumulus cells surrounding the tubal eggs of the rat are embedded was described by Long (1912) and according to Pincus and Enzmann (1935) this substance is removed by a heat-labile substance produced by the spermatozoa.

Ovulation (McClean and Rowlands, 1942) was produced by Rowlands' method and the action of hyaluronidase from various sources and other reagents on the gel and cell masses round the egg was observed by a simple technique which involved recording the time taken by the various test preparations to produce complete denudation of the egg when all the cells had fallen away to the bottom of a watch-glass. Plate XVIII, Figure 1[1] shows (a) a collection of several ova surrounded by the cumulus cell mass of the corona radiata, (b) a similar group of ova immediately after treatment with hyaluronidase; the eggs are floating freely and the cells in the background have fallen to the bottom of the watch-glass, and (c) a few ova in which the gel is almost completely dissolved but in which its outline and a few remaining adherent cells can be seen. The results obtained are recorded in Table 2 together with the hyaluronidase activity of the enzyme preparations tested.

The rate at which the cumulus cells are dispersed is closely correlated with the concentration of hyaluronidase, obtained not only from spermatozoa and testis but also from streptococcus, *Cl. welchii* and *Cl. septicum* and this is perhaps more clearly demonstrated in the accompanying graph (Figure 2). The activity of the enzyme derived from snake venom and from bacteria is additional evidence that this is a specific activity of the

[1] This plate will be found facing page 272.

TABLE 2. The action of hyaluronidase and other substances and preparations on the dispersion of the cumulus cells of the rat ovum

Substance tested	Hyaluronidase		Time taken to disperse cumulus cells and corona radiata (min)
	v r.u./ml	m c.p test	
Bovine testis hyaluronidase	0 6		165
	0 85		142
	6 0		36
	8·5		33
	15 0		20
	15 0		20
	30·0		17
	60 0		11
	85·0		9
Streptococcal hyaluronidase	0 6		100
	6 0		32
Cl. welchii hyaluronidase	5–7·5		27
	10–15		19
Vibrion septique hyaluronidase	2 5		30
Rattlesnake venom			
0 5%[1]		+	17
0 05%		+	27
Rat sperm extract[1]		+	12
	3 25		22
Rabbit semen extract[1]		+	18
Rat and guinea-pig vesicular fluid		–	No effect in 2 hr.
Rat prostatic fluid		–	No effect in 2 hr.
Taka diastase, 0 5%		–	78
Diastase, 0 5%		–	No effect in 3 hr
Sodium glycocholate, 0 5%		–	No effect in 3 hr.
Baker's solution		–	No effect in 3 hr.
Physiological salt solution		–	No effect in 3 hr

[1] Ten minutes' heating at 100° C destroyed activity as shown both by m c p test and inability in a period of 3 hours to disperse the cells surrounding the egg

enzyme, since it is unlikely that preparations from such diverse sources would all be contaminated with some other active agent. Cell masses exposed to the action of detergents such as sodium glycocholate become almost invisible but the gel is not liquefied. Vesicular and prostatic secretions have no action on the gel. The circumstantial evidence provided by these experiments suggested that the gel surrounding the eggs is composed

of hyaluronic acid and that the activity of the enzyme might explain the enormous numbers of spermatozoa apparently necessary to secure a maximum rate of fertilization. It may be that a large number of spermatozoa are required in the vicinity of the egg to produce a sufficient concentration of hyaluronidase to liquefy the gel and so allow penetration by the single effective

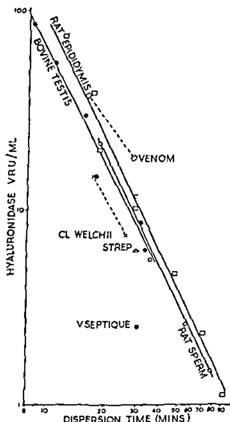


FIG. 2

sperm. Our results with rat ova were confirmed in mice by Fekete and Duran-Reynals in 1943. Later, Rowlands (1944) reported increased fertility in rabbits following insemination with diluted sperm suspensions to which hyaluronidase had been added. This work has not, however, been confirmed.

There is a close correlation between hyaluronidase content and sperm density in the semen of man, rabbit, bull and boar, but in dogs and fowls no such correlation can be found (Swyer, 1947a, b). The discrepancy in the dog is unexplained, but it is interesting to note that according to Brambell (1925) bird ova have no cumulus cell mass, there is also little hyaluronidase in bird testis and none in fowl tumours. Swyer also found that most of the hyaluronidase in semen is associated with the spermatozoa, the remainder being in solution in the plasma; the spermatozoa do not actively produce hyaluronidase, but they liberate the preformed enzyme at a rate determined by the concentration of hyaluronidase in their environment. This explains the release of enzyme in the Fallopian tubes in a concentration sufficient to disintegrate the cumulus cell masses. Austin (1948) cast some doubt on the hypothesis that destruction of the gel supporting the cumulus cell mass is a necessary precursor of fertilization in all species since he had been able to find sperms within the vitellus with visible male and female pronuclei in eggs still surrounded by a cumulus cell mass. He conceded, however, that the local presence of the enzyme might assist the passage of the individual sperm.

There are two major social problems connected with fertilization—infertility and excessive fertility. The first is often an extremely distressing personal problem and any increase in knowledge that improved the treatment of individual cases would be welcome. Even if it could be proved that any individual cases of infertility were due to a deficiency in the hyaluronidase carried by the spermatozoa it is unfortunate that, so far, nobody has overcome the technical difficulty of getting added hyaluronidase where it may be wanted, that is at the site of fertilization.

When we turn to excessive fertility and the need for an effective and aesthetically satisfactory means of controlling conception, the position may be more hopeful. In the chain of events leading to the implantation of the zygote in the uterus it appears that hyaluronidase may be essential in the actual fertilization of the ovum. If an effective inhibitor of this enzyme, with no undesirable side-effects, could be provided

at the site of fertilization a major advance in contraceptive technique might be achieved. If sufficient concentration of such an inhibitor could be obtained following oral administration this would obviously be far preferable to any agent that had to be inserted into the vagina. A considerable amount of work has already been done on hyaluronidase inhibitors starting with Hadidian and Pirie's (1948) observation that nitrated hyaluronic acid inhibited the enzyme and Pincus, Pirie and Chang (1948) who found that it inhibited the dispersal of cumulus cells *in vitro* and acted as a contraceptive when put into rabbits' vaginas before mating. The work of hyaluronidase inhibitors has been ably reviewed recently by Mathews and Dorfman (1954). It may be said, I think, that so far all of the experiments in animals on the inhibition of fertilization have given results consistent with the view that hyaluronidase plays an important role in fertilization, but one must remember that reagents that inhibit hyaluronidase may also upset some other important factor in this delicately adjusted process.

The first claim to have obtained a hyaluronidase-inhibitor that was effective as a contraceptive by the oral route in mice and rats was made by Martin and Beiler (1952). They used a phosphorylated hesperidin. This was followed by a report from Sieve (1952) in which he claimed a quite astonishing success with 300 human couples who took regular divided daily doses of the same phosphorylated hesperidin orally for periods up to 30 months. The only two recorded pregnancies were due to admitted failure to take the drug regularly. Unfortunately, these authors have not so far been able to repeat this work. In conversation, Dr. Martin informed me that he was convinced of the validity of his own results in animals and of Sieve's in man, but he thought their subsequent failures were due to their inability to reproduce exactly the same phosphorylated hesperidin as the specimen originally used for this work. Thus although a disappointing rebuff has to be admitted, the position is hopeful and there are indications that this search for a suitable inhibitor is on the right lines.

Finally I want to draw your attention to the following quotation from the report of the Royal Commission on Population

in 1949: 'Control by men and women over the numbers of their children is one of the first conditions of their own and the community's welfare and in our view mechanical and chemical methods of contraception have to be accepted as part of the modern means, however imperfect, by which it can be exercised.'

They apparently accepted the imperfection of available methods but they do imply that it is as vital to control world population as it is to avoid mutual destruction with nuclear weapons and to find a solution of the problems of living peacefully together; indeed it is part of the same problem I want to endorse Pirie's excellent summary of the situation (1952a) and his trenchant appeal (1952b) for the extension of basic research into the physiology of the gametes. As he says, this research is seriously hampered by lack of funds, probably because it is not realized that the problem is as urgent as the improvement of war potential. He also points out the intrinsic interest to scientists of this subject and the improvement of available techniques for its study. The gametes would also be very suitable raw material for much research into metabolic problems. As Pirie pertinently remarks: 'The strategy of getting applied research done, when it is obvious that it cannot usefully be started till we have more fundamental knowledge, is to see that people who are already engaged in fundamental research sometimes use the material in which we have a practical interest.'

Such basic research with adequate finance into all the aspects of spermatogenesis, ovulation, fertilization and the implantation of the ovum is essential to the satisfactory solution of the urgent social problem posed by the Royal Commission.

REFERENCES

AUSTIN, C. R (1948). *Nature, Lond* 162, 63
BORDET [illegible]
BOYLA
BRAMB
CHAIN
CHAIN

CLAUDE, A. (1937). *J. exp. Med.* 66, 353.
COWAN, M. L. (1922). *Brit. J. exp. Path.* 3, 187.
DURAN-REYNALS, F. (1928a). *C. R. Soc. Biol.* 99, 6.
DURAN-REYNALS, F. (1929). *J. exp. Med.* 50, 327.
DURAN-REYNALS, F. (1933). *J. exp. Med.* 58, 161.
DURAN-REYNALS, F. (1939). *J. exp. Med.* 69, 69.
DURAN-REYNALS, F. and STEWART, F. W. (1931). *Amer. J. Cancer*, 15, 2790.
DURAN-REYNALS, F. and SUNER PI, J. (1928b). *C. R. Soc. Biol.* 99, 1908.
[illegible]
HAHN, L. (1946a). *Ark. Kemi. Min. Geol.* 22A, No. 1.
HAHN, L (1946b). *Ark. Kemi. Min. Geol.* 22A, No. 2.
HALE, C. W. (1944a) *Biochem. J.* 38, 362.
HALE, C. W. (1944b). *Biochem. J.* 38, 368.
HARE, R. (1931). *Brit. J. exp. Path.* 12, 261.
HIRST, G. K. (1941). *J exp. Med.* 73, 493.
[illegible] *J. exp. Med.* 53, 387.
HUMPHREY, J H. (1944). [illegible]
HUMPHREY, J. H and JACQUES, R (1953). *Biochem. J* 53, 59.
KASS, E. R. and SEASTONE, C. V. (1944). *J. exp. Med.* 79, 319.
KENDALL, F. E , HEIDELBERGER, M. and DAWSON, M. H. (1937). *J. biol. Chem.* 118, 61.
LACK, C. H. (1948). *Brit J. exp. Path.* 29, 191.
LANCEFIELD, R. C. (1928). *J exp. Med* 47, 91.
LONG, J A. (1912). *Univ. Calif. Publ. Zool.* 9, 105.
McCLEAN, D (1930). *J Path Bact.* 33, 1045
McCLEAN, D (1931). *J Path. Bact* 34, 459
McCLEAN, D. (1936) *J Path. Bact* 42, 477.
McCLEAN, D. (1941a) *J. Path Bact.* 53, 13.
McCLEAN, D. (1941b). *J Path. Bact.* 53, 156
McCLEAN, D. (1942). *J Path Bact* 54, 284.
[illegible] *J.* 35, 159
[illegible], ii, 434.
[illegible] W. (1943). *Lancet*, i, 355.
[illegible] *ature, Lond.* 150, 627.
MACLENNAN, A. P. (1956a). [illegible] 14, 134.
MACLENNAN, A. P. (1956b) *J Gen Microbiol* 14, 143.
MADINAVEITIA, J., TODD, A. R., BACHARACH, A. C. and CHANCE, M. R. A (1940). *Nature, Lond.* 146, 197.

MADINAVEITIA, J. and QUIBELL, T. H. A. (1940). *Biochem. J.* **34**, 625.
MARTIN, E. J. and BEILER, J. M. (1952). *Science*, **115**, 402.
MATHEWS, M B and DORFMAN, A (1954) *Connective Tissue in Health and Disease* Ejnar Munksgaard, Copenhagen.
MEYER, K, HOBBY, G., CHAFFEE, E. and DAWSON, M. H. (1940a). *J. exp. Med.* **71**, 137.
MEYER, K, HOBBY, G., CHAFFEE, E. and DAWSON, M. H. (1940b). *Proc. Soc. exp Biol, N.Y.* **44**, 294.
MEYER, K. and PALMER, J. W (1934). *J. biol. Chem.* **107**, 629
MEYER, K. and PALMER, J. W (1936) *J. biol. Chem* **114**, 689

em. **19**, 388.
PIRIE, N. W. (1952a). *Eugenics Review*, **44**, 129
PIRIE, N. W. (1952b) *Lancet*, **1**, 54.
ROBERTSON, W. VAN B., ROPES, M. W. and BAUER, W. (1940). *J. biol. Chem.* **133**, 261.
ROGERS, H J. (1945). *Biochem. J.* **39**, 435.
ROGERS, H J (1946a). *Biochem. J.* **40**, 583.
ROGERS, H. J. (1946b) *Biochem. J.* **40**, 782
ROGERS, H. J (1948). *Biochem J.* **42**, 633.
ROTHBARD, S (1948). *J exp. Med.* **88**, 325.
ROWLANDS, I. W. (1942) *Nature, Lond.* **150**, 267.
ROWLANDS, I. W. (1944). *Nature, Lond.* **154**, 332.
Royal Commission on Population (1949) Report. H.M S O. Cmd. 7695.
SEASTONE, C V. (1934). *J Bact.* **28**, 481.
SEASTONE, C. V. (1939) *J. exp Med* **70**, 361.
SIEVE, B F. (1952) *Science*, **116**, 373.
SWYER, E I. M. (1947a) *Biochem. J* **41**, 409
SWYER, E. I. M. (1947b). *Biochem. J.* **41**, 413

CLAUDE, A. (1937). *J. exp. Med.* 66, 353.
COWAN, M. L. (1922). *Brit. J. exp. Path.* 3, 187.
DURAN-REYNALS, F. (1928a). *C. R. Soc. Biol.* 99, 6.
DURAN-REYNALS, F. (1929). *J. exp. Med.* 50, 327.
ed. 58, 161.
ed. 69, 69.
W. (1931). *Amer. J. Cancer.* 15, 2790.

HAHN, L. (1945a). *Ark. Kemi. Min. Geol.* 19A, No. 33.
HAHN, L. (1945b). *Ark. Kemi. Min. Geol.* 21A, No. 1.
HAHN, L. (1946a). *Ark. Kemi. Min. Geol.* 22A, No. 1.
HAHN, L. (1946b). *Ark. Kemi. Min. Geol.* 22A, No. 2.
HALE, C. W. (1944a). *Biochem. J.* 38, 362.
HALE, C. W. (1944b). *Biochem J.* 38, 368.
HARE, R. (1931). *Brit. J. exp. Path.* 12, 261.
HIRST, G. K (1941). *J. exp. Med* 73, 493.
HOFFMAN, D. C and DURAN-REYNALS, F. (1931). *J. exp. Med.* 53, 387.
HUMPHREY, J H. (1943a) *Biochem. J.* 37, 177.

J. 53, 59.
ed. 79, 319
KENDALL, F. E., HEIDELBERGER, M. and DAWSON, M. H (1937). *J biol. Chem.* 118, 61.
LACK, C. H. (1948). *Brit J. exp. Path.* 29, 191.
LANCEFIELD, R C. (1928). *J. exp. Med.* 47, 91.
LONG, J A. (1912). *Univ Calif Publ. Zool.* 9, 105.
MCCLEAN, D (1930). *J. Path Bact.* 33, 1045.
MCCLEAN, D. (1931). *J. Path. Bact.* 34, 459
MCCLEAN, D (1936). *J. Path. Bact.* 42, 477
MCCLEAN, D. (1941a) *J Path. Bact.* 53, 13.
MCCLEAN, D. (1941b) *J Path Bact* 53, 156.
MCCLEAN, D. (1942). *J Path. Bact* 54, 284.

MACLENNAN, A. P. (1956a). *J Gen. Microbiol.* 14, 134.
MACLENNAN, A. P. (1956b). *J. Gen. Microbiol* 14, 143.
MADINAVEITIA, J., TODD, A. R., BACHARACH, A. C. and CHANCE, M. R. A (1940). *Nature, Lond.* 146, 197.

the posterior pituitary was intact. Fisher, Ingram and Ransom's monograph (1938) summarizing their views on the essential unity of the posterior lobe-hypothalamic system had not yet appeared.

Since 1935 we have acquired a great deal more information and some things are clearer, but we still do not know exactly how or in what form the active substances are made.

Site of Production of Posterior Lobe Hormones

In order to make some of the following points clearer Figure 1 shows in diagrammatic form the posterior lobe of the pituitary and the two paired hypothalamic nuclei with which it is most intimately connected.

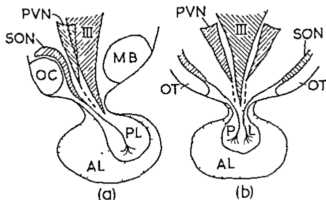


Fig 1 Diagram of hypothalamus and pituitary. (*a*) Saggital section, (*b*) coronal section PL, posterior lobe, AL, anterior lobe, III, third ventricle; OC, optic chiasma, OT optic tract, MB, mammillary body, SON, supraoptic nucleus; PVN, paraventricular nucleus

In the past, when considering where and how the posterior lobe hormones were made, there was the difficulty that extraction of the lobe yielded potent substances (there was good reason to believe that the substances did come from the posterior lobe and not from the pars intermedia), yet the structure of this lobe was quite unlike that of other glands It appeared to consist wholly of nerve endings, blood vessels and neuroglia. Gersch (1939) put forward the view that the neuroglia, or pituicytes, were the

XVII

Release and Activity of Posterior Pituitary Hormones

MARY PICKFORD

THERE is a great deal of interest to be had from reading old text books. The really old ones will obviously express ideas other than our own, but early editions of present-day standard works bring it home how much our knowledge of detail has changed. In the 1923 edition of Macleod's *Physiology in Modern Medicine* it is stated that removal of the pituitary body leads to death. With regard to the posterior lobe some stress is laid on the action of extracts on smooth muscle, and particularly on their blood-pressure raising effects. There is no discussion of the effect on uterine muscle, but a figure shows a record of uterine contraction. Extracts are said to cause a remarkable increase in urinary flow. There is a good factual account of their galactogue action. In the 1935 edition the uterus is said to contract in a manner unconditioned by its state. There is, too, the statement that the chief action on the kidney is an antidiuretic one, and, further, that certain types of hypothalamic injury, or ablation of the posterior lobe, give rise to polyuria. By that time Cushing (1932) had made his observations on the effect of injecting posterior lobe extracts into the cerebral ventricles. Abel (1923) and Kamm and his co-workers (1928) had produced their opposing ideas, namely, that the hormones were part of a single parent molecule, or that they were formed separately from each other. Verney (1926) had published his observations on the antidiuretic substance picked up by blood flowing through a head in which

PLATE XIX

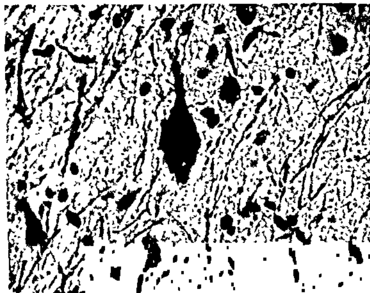


FIG 2 Cell of supraoptic nucleus of dog, stained haematoxylin chrome-alum phloxin Cell and axons dark with granules (× 500)

actual site of hormone manufacture. This idea was not entirely satisfactory since, for one thing, many more nerve ends reach the posterior lobe than there are pituicytes to innervate. Also, there is no evidence that other glial cells ever secrete active substances, not that this is necessarily any argument. Another possibility was that the hormones were made by the nerve ends and released into the circulation. This was a suggestion different from, and not to be confused with, the more recent one on the neurosecretory origin of the active substances.

This more recent view has much evidence to support it and can explain a number of the facts. Some years ago E. and B. Scharrer (1945, 1954) showed what appeared to be secretory granules and droplets in nerve cells of both invertebrates and vertebrates. It is accepted that in invertebrates such as worms, insects and crustacea certain nerve cells quite clearly have an endocrine function, often related to reproductive and developmental activity. Thus, it was not a completely new conception when a group of German workers (Bargmann, 1949; Bargmann and Hild, 1949; Ortmann, 1950, 1951; Zetler, 1953) put forward the idea of neurosecretion in the hypothalamic-posterior lobe system. They used Gomori's chrome alum haematoxylin phloxin stain and noticed that the supraoptic and paraventricular cells, their processes and the posterior lobe contained dark-coloured granules not seen elsewhere in the central nervous system (Plate XIX, Figure 2). In the cell bodies the fine granules were scattered about the cytoplasm. The axons contained the stainable matter in beaded form inside the sheath and close to the axoplasm. It was present in the dendrites too. In the posterior lobe the staining material always lay near the blood vessels. Some of it could also be seen close to the ependyma of the recess of the third ventricle. This stainable material is often referred to as Gomori substance. Experiment established the following facts. In animals that have been well hydrated before death the supraoptic cells and posterior lobe are loaded with Gomori substance. In severely dehydrated animals the Gomori substance may be markedly reduced or absent. If animals are dehydrated and then rehydrated before death Gomori substance is present

in quantities depending on the length of time since dehydration ended. If the pituitary stalk is cut at a high level or if the supra-optico-hypophysial tract is sectioned, the Gomori substance disappears from the parts below the section, and above the section from those nerve cells which undergo retrograde degeneration, as many of them do. Any nerve cells and stubs of axons that survive are so laden with the substance that they stain almost black and their fine structure is concealed (Plate XX, Figure 3).

Clearly, then, the amount of Gomori substance varies with the state of the animal, and varies, moreover, in the sense that might be anticipated supposing this material is connected directly with antidiuretic hormone (ADH). Thus, in dehydration when there must be great need for circulating ADH for water conservation, the cells and posterior lobe are drained of this material. In states of hydration when there is no special call for ADH plenty of Gomori substance can be seen.

Minute Anatomy of Posterior Lobe

Until recently the minute anatomy of the posterior lobe has been obscure. Bodian (1951) produced an illuminating account of the relationships of nerve ends, blood vessels and neuroglia. It had been appreciated that sometimes in some parts a vaguely lobular pattern could be discerned. Bodian found that in the opossum the lobular pattern was unmistakable and, with this point clear, it was then easier to understand the variations and compressions of the type in other vertebrates. He found that in the opossum the supraoptic nerve fibres enter each lobule in small bundles, fan out, and ultimately split into fibrils which lie directed towards the periphery of the lobule in close relation to the surrounding capillaries. The bodies of the pituicytes are in the centre of the lobules and their processes stretch down between the nerve fibrils, also towards the capillaries. Like the German workers he saw the Gomori substance lying inside the nerve sheaths in beads. Here and there he saw larger blebs, probably forming the structures seen by Herring and named after him the Herring bodies. Of particular interest is Bodian's description of the concentration of Gomori substance round the

Plate XX

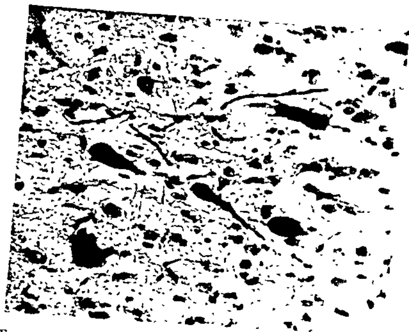


Fig. 8. Para ...

The absence of diabetes insipidus after simple posterior lobe removal brings out another point, namely that the posterior lobe is not an essential sensory organ (a suggestion that has been made) responding to variations in water concentration of the blood, and adds weight to Verney's suggestion that osmoreceptors, i.e. structures which can be activated by changes in blood osmotic pressure, lie somewhere in the anterior hypo-

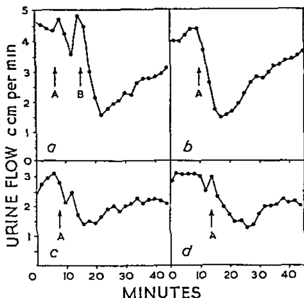


FIG 4 Water diuresis curves of conscious dog, responses to injections, *a* and *b* 5 days before, *c* and *d* 11 days after, removal of posterior lobe of pituitary. *a* at B, injection of 21 ml of 2 5% NaCl into right carotid in 20 sec, *b*: at A, 1 mU of postpituitary extract injected intravenously in 15 sec, *c* at A, injection of 21 ml of 2 5 + NaCl into right carotid in 20 sec.; *d* at A, 0 1 mU postpituitary extract injected intravenously in 20 sec. (From Verney, 1946.)

thalamus (Verney, 1947). His observations were made on conscious dogs provided with denervated carotid loops. In some of the animals the external carotid was ligated. In this way, on injecting solutions into the carotid artery Verney could be sure that a fair concentration of injected substance reached the central nervous system but was well diluted before it came to

ends of the nerve fibrils, looking very like thick finger stalls covering the ends of the thin nerve fingers.

So far, then, the argument runs that the posterior lobe hormones are made in the supraoptic and paraventricular cells, reach the posterior lobe for storage and are released on demand into the blood stream. Thus, these neurones act both as endocrine organs and as nerve cells and fibres carrying impulses.

Release of Hormones

In order to account for the known facts this idea must be elaborated and modified. Observations by Trendelenburg (1928) and Sato (1928) showed that the vasopressor and oxytocic activity of hypothalamic extracts is increased by hypophysectomy. It could be that following removal of the pars nervosa the neurones persist in making hormones, but being unable to discharge them to the usual store-house increase their own concentration to saturation point. This could account for the great increase in Gomori substance seen after hypophysectomy. Further, it has been known for some time that if the posterior lobe is removed, but the hypothalamus not directly interfered with, diabetes insipidus may be far from maximal, or even not appear at all. This suggests that the hormones can reach the blood stream direct without the help of the posterior lobe, unless there is another unidentified mechanism for the regulation of water excretion. Certainly the blood supply of the supraoptic and paraventricular nuclei is great enough for direct release to be possible, even if this is not the usual route of discharge. In favour of this last idea are Keller's (1942) observations. In survival experiments on cats and dogs he entirely isolated the median eminence and posterior lobe from the hypothalamus so that they completely atrophied, yet only rarely was there any sign of diabetes insipidus. In the few instances where diabetes did occur all supraoptic cells had disappeared. This is in accord with the observations of many other workers that more than 90 per cent of supraoptic cells must atrophy before diabetes insipidus becomes either apparent or latent. These points are certainly in favour of a direct discharge of ADH into the blood from the nerve cells.

large number of drugs can also cause antidiuresis, e.g. certain anaesthetics, nicotine, morphine, ferritin, cinchoninic acid, acetylcholine and adrenaline and noradrenaline (Pickford, 1942). The last two substances affect urine flow in more than one way.

Acetylcholine (ACh) appears to act locally on the supraoptic cells, at any rate it is more effective when given by intracarotid

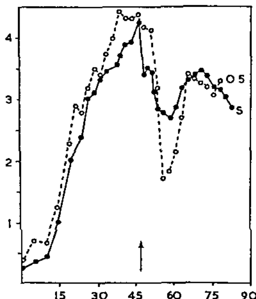


FIG 6. Water diuresis curves of conscious dog Estimation of the amount of antidiuretic substance liberated in response to 40 sec emotional stimulus of the same strength and with the same bitch as in Fig 5, but 1 month after removal of the posterior lobe. The inhibition resulting from the emotional stimulus (graph S) is compared with that from 0 5 mU postpituitary extract injected during the curve of the previous day (graph 0 5) Ordinate and abscissa as in Fig 5 (From O'Connor and Verney, 1942.)

than intravenous injection (Pickford and Watt, 1951), and also acts when injected in very small amounts direct amongst the supraoptic cells (Figures 7 and 8) (Pickford, 1947). In the conscious animal its action is prevented by the previous application of an anticholinesterase to the nerve cells (Duke, Pickford and Watt, 1950) If ACh acts at this site then so, too, should nicotine

the peripheral organs. He used iso- or hypertonic solutions of such substances as NaCl, Na_2SO_4, glucose, sucrose and urea. Those substances exerting an osmotic pressure and which are not freely permeable through cell membranes caused an inhibition of water diuresis (Figure 4). Freely permeable substances like urea had no effect. Antidiuresis could also be produced if much greater amounts of the active substances were injected

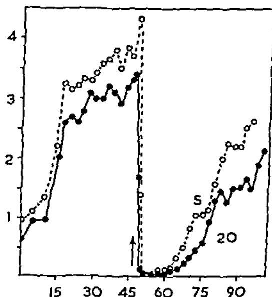


Fig. 5 Water diuresis curves of conscious dog. *Estimation of the amount* [illegible]

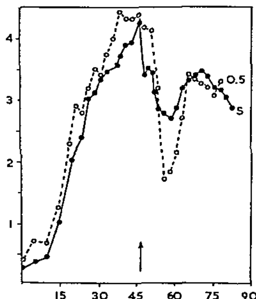
from the intravenous injection of 20 mU postpituitary extract (graph 20). Ordinate, the rate of urine secretion over the interval before the plotted point Abscissa, time, minutes (From O'Connor and Verney, 1942.)

by vein, but the effects were less striking and less immediate. *After posterior lobe removal the antidiuretic effects* were markedly reduced

Antidiuresis can occur in response to various types of stimulation. As O'Connor and Verney (1942) have shown emotion can be strongly antidiuretic and the response depends on the presence of a functioning posterior lobe (Figures 5 and 6). A

large number of drugs can also cause antidiuresis, e g. certain anaesthetics, nicotine, morphine, ferritin, cinchoninic acid, acetylcholine and adrenaline and noradrenaline (Pickford, 1942). The last two substances affect urine flow in more than one way.

Acetylcholine (ACh) appears to act locally on the supraoptic cells, at any rate it is more effective when given by intracarotid



[illegible]

extract injected during the curve of the previous day (graph 0 5) Ordinate and abscissa as in Fig. 5 (From O'Connor and Verney, 1942)

than intravenous injection (Pickford and Watt, 1951), and also acts when injected in very small amounts direct amongst the supraoptic cells (Figures 7 and 8) (Pickford, 1947). In the conscious animal its action is prevented by the previous application of an anticholinesterase to the nerve cells (Duke, Pickford and Watt, 1950). If ACh acts at this site then so, too, should nicotine

in a suitable dose. Burn, Truelove and Burn (1945) showed that smoking with inhalation, and also the intravenous injection of nicotine, were powerfully antidiuretic in both man and animals, and that antidiuresis was not seen in animals from which the

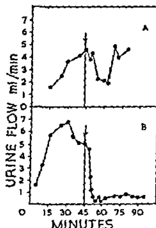


Fig. 7. Water diuresis curve of conscious dog A, at arrow 3 mg. ACh injected intravenously B, at arrow, 0 5 mg ACh injected into the left carotid artery (From Pickford and Watt, 1951.)

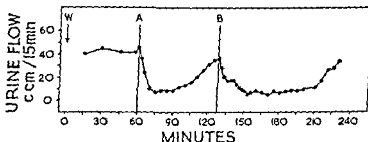


Fig. 8. Water diuresis curve of chloralosed dog At A, 7 μg ACh injected into the left supraoptic nucleus At B, 7 μg ACh + 2 μg eserine salicylate injected into the right supraoptic nucleus (From Pickford, 1947)

posterior lobe had been removed (Figure 9). Morphine, also, seems to act directly on the supraoptic cells since it is effective not only when injected intravenously but also when applied direct in small amounts to the neurones (Figure 10). It differs from

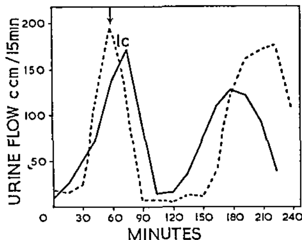


Fig 9 The subject drank 1 litre of water at zero The broken line is an experiment in which 0 5 mg. nicotine (base) was injected intravenously at the arrow. The solid line shows the effect of smoking one cigarette in the same subject. (From Burn, Truelove and Burn, 1945)

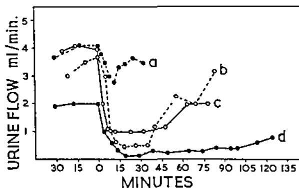


Fig. 10 Water diuresis curves of chloralosed dogs, the injection of morphine sulphate into the supraoptic nuclei (4 experiments) The curves have been placed so that in each case the injection was given at zero time on the graph. Doses: *a*, 4 μg; *b*, 8 μg; *c*, 16 μg; *d*, 32 μg. (From Duke, Pickford and Watt, 1951.)

ACh in that it is antidiuretic even when the supraoptic cells are under the influence of an anticholinesterase (Duke, Pickford and Watt, 1951). Thus, whatever its action may be it is not exclusively a cholinergic one. Morphine, in addition, causes the supraoptic cells to lose Gomori substance (Abrahams, unpublished).

Adrenaline and noradrenaline have a triple and somewhat complex action. O'Connor and Verney (1945) observed that in some, but not all, of their conscious animals the antidiuretic response to emotion might be very short-lived and look like the

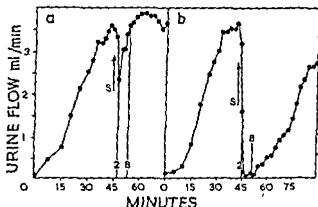


Fig. 11. Water diuresis curves of conscious dogs *a*, at S, 60 sec. emotional stimulus, *b*, at S, same stimulus 1 month after denervation of kidneys and suprarenals. (From O'Connor and Verney, 1945.)

effect of injecting a small dose of adrenaline, as against the longer-lasting and posterior lobe type of antidiuresis which was seen in other animals. Some dogs varied in their response and on one day showed a short, and on another a long-lasting, inhibition. It looked as though sympathetic activity sometimes interfered with the antidiuretic response. O'Connor and Verney, therefore, cut the splanchnic nerves and performed lumbar sympathectomy in those dogs which always showed only the short inhibition. After operation these animals now always gave a prolonged inhibition of water diuresis in response to emotional stress (Figure 11). Further, they found that a small intravenous dose of adrenaline injected half a minute before the stress

stimulus prevented the slow posterior lobe inhibition, but did not affect the response of the kidney to injected posterior lobe extract. To summarize these results, individuals vary in their response to an emotional stimulus, some showed antidiuresis of posterior lobe origin, some only an adrenaline type of inhibition, but these last, after sympathectomy, showed a posterior lobe inhibition. In these individuals the suppression of the posterior lobe antidiuresis was of central, not peripheral, origin, and appeared to be in no way related to changes in general blood pressure.

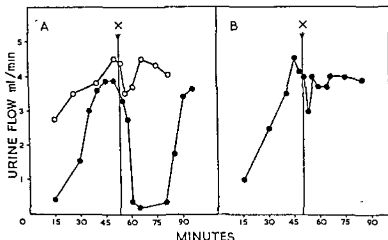


FIG 12 Water diuresis curves in a conscious dog The effect of intracarotid injections given at X In A, •—• 200 µg ACh, o—o, 2 µg adrenaline HCl In B, •—• 2 µg adrenaline HCl followed 11 secs later by 200 µg ACh

Now, if the antagonism between adrenaline and emotion with regard to antidiuresis is central, and if ACh causes antidiuresis by a central action, then one should find antagonism between adrenaline and ACh Such indeed is the case. If observations are made on conscious dogs during water diuresis and the injections made into the carotid artery then there is 100 per cent antagonism between these two drugs, providing they are given within certain limits of dose and time (Figure 12). Thus, 1–3 µg. adrenaline always annuls the normal antidiuretic effect of 200 µg ACh if the adrenaline is injected from

8 to 45 seconds before the ACh (Abrahams and Pickford, unpublished). Interpretation of these results is not easy because Dearborn and Lasagna (1952) and Eränko and Karvonen (1952) simultaneously showed that large amounts of adrenaline given intravenously caused an inhibition of urine flow which was not seen after posterior lobe removal, i.e. adrenaline can in some circumstances actually release ADH. Finally, adrenaline can alter the rate of urine flow by a direct vascular action.

Relation between Vasopressin and Oxytocin

A further problem is the relation between oxytocin and ADH. It is still uncertain whether the two active substances, now known to be small polypeptides (Vigneaud *et al.*, 1954a and b), are attached to a single parent molecule or whether they are made separately from each other. Regarding the Gomori substance, a recent paper describes how, if histological observations are made at set times after suitable stimulation, this substance appears to move towards the capillaries of the posterior lobe, and that certain dark granules seen in the blood vessels may even be this substance after discharge from the pituitary (Rothballer, 1953). This would agree with the idea of a large parent molecule. On the other hand, the Gomori substance can apparently be dissolved out with lipid solvents leaving the active materials behind. This suggests that the Gomori substance is a carrier for the hormones (Zetter, 1953). However, there are further difficulties. If extracts of posterior lobe are assayed for antidiuretic and oxytocic activities, the hormones are found to be present in a roughly 1/1 or 0·5/1 ratio according to the species from which the gland is taken (Dicker and Tyler, 1953a). If the activity of (dog) anterior hypothalamus is assayed considerable antidiuretic but little or no oxytocic material is found (Dicker and Tyler, 1953b; Hild and Zetler, 1951; Vogt, 1953). This finding suggests that the two hormones are not made in the same part of the posterior lobe system. Then, Dicker and Tyler (1953a and b) have assayed the posterior lobe after parturition and lactation and found a marked reduction in oxytocin relative to vasopressin. This suggests that the release and manufacture of these two substances do not run parallel. This last

point might be expected. ADH and oxytocin are not always in demand at the same moment and it seems reasonable that they should be made and released independently, but at present we have little idea how this can be brought about. The nerve fibres entering the posterior lobe all look alike and all are gloved by Gomori substance, and whatever stimulus has been used to activate the posterior lobe, always both hormones are liberated into the circulation, though not in equal amounts. Haterius and Ferguson (1938) and Harris (1947) used electrical stimulation of hypothalamus, stalk or posterior lobe, the latter worker on conscious animals. By observing the uterine movements and urine flow Harris noted that about 1 part vasopressin was released to about 4–10 parts oxytocin. Cross (1951) found that in rabbits the suckling stimulus released 1 part vasopressin to about 100 parts oxytocin. In dogs stimuli designed to liberate ADH at the same time released 15–20 times as much oxytocin (Abrahams and Pickford, 1954). These varied results all indicate that there must be some measure of independence for the two hormones, though it is by no means complete. There has recently been a note to the effect that the paraventricular nuclei may be closely concerned with oxytocin manufacture (Olivecrona, 1954).

Oxytocin

Finally, something should be said about the action of oxytocin on the uterus. It now seems clear that this substance is a true hormone for the purpose of the let down or ejection of milk. Not everyone yet believes that it is necessary for good uterine contraction in parturition It is said that certain animals can give birth normally after posterior lobe removal (Smith, 1932), and that in humans, providing pregnancy does occur, diabetes insipidus is not a bar to natural birth Regarding the animals, a sufficient number of neurosecretory cells may have survived to pass hormone direct into the blood. With clinical material, unless the polyuria is maximal, how can one be sure that there are no secreting cells present? Also, Marañon (1947) has described the case of his patient with diabetes insipidus who suffered uterine atony in repeated pregnancies and only success-

fully bore her child when she was treated with posterior lobe extracts. In dogs it is clear that the posterior lobe has a striking influence on the uterus. In a series of experiments the animals were provided with denervated carotid loops and a fistula of one uterine horn. Thus, urine flow and uterine activity could be simultaneously observed, the latter by means of a small balloon inserted through the fistula. Sometimes the ovaries were removed at the time the fistula was made. This meant

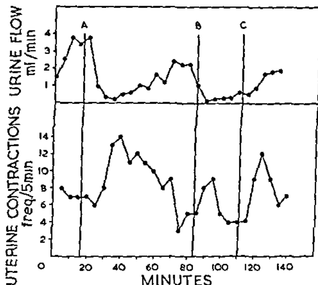


FIG. 13. Effect of intracarotid injection of NaCl on urine flow and uterine motility in the conscious dog Upper record, urine flow. Lower record, frequency of uterine contraction. At zero time 350 ml water by mouth At A, 2 4 ml 1 7 M. NaCl solution by intracarotid injection in 5 sec At B, 5 mU Pitressin intravenously At C, 5 mU Pitocin intravenously. (From Abrahams and Pickford, 1954.)

that for about the next six weeks the uterus showed a high degree of spontaneous activity. If the ovaries were left in, the uterine movements were minute. Now, if Verney's stimulus to antidiuresis, viz., intracarotid injections of hypertonic NaCl solution, was used, then always there was increased uterine activity beginning and ending at the same time as the antidiuresis (Figure 13). The results were exactly similar whether the

uterine contractions were large or small. In terms of injected posterior lobe extract, the response was equivalent to less than 5 mU vasopressin and 80–100 mU oxytocin (Abrahams and Pickford, 1954). It was from experiments such as these that the figures were obtained about the unequal amounts of these two hormones released by a single stimulus. The behaviour of the

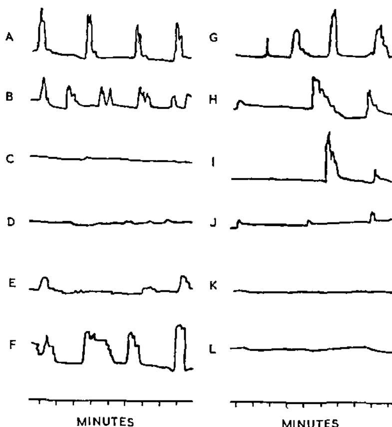


FIG. 14 Records of spontaneous uterine contractions before and after induction of experimental diabetes insipidus A, normal, 27 May 1953 [illegible]

[illegible], 12 June (From Abrahams and Pickford, 1954)

fully bore her child when she was treated with posterior lobe extracts. In dogs it is clear that the posterior lobe has a striking influence on the uterus. In a series of experiments the animals were provided with denervated carotid loops and a fistula of one uterine horn. Thus, urine flow and uterine activity could be simultaneously observed, the latter by means of a small balloon inserted through the fistula. Sometimes the ovaries were removed at the time the fistula was made. This meant

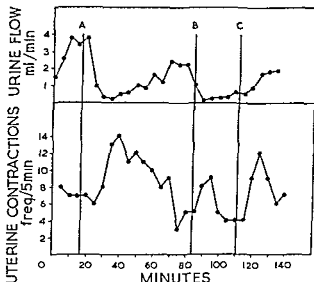
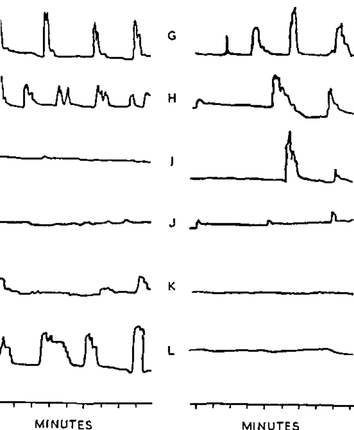


FIG. 13 Effect of intracarotid injection of NaCl on urine flow and uterine motility in the conscious dog. Upper record, urine flow. Lower record, frequency of uterine contraction. At zero time 350 ml water by mouth. At A, 2.4 ml 1.7 M NaCl solution by intracarotid injection in 5 sec. At B, 5 mU Pitressin intravenously. At C, 5 mU Pitocin intravenously. (From Abrahams and Pickford, 1954.)

that for about the next six weeks the uterus showed a high degree of spontaneous activity. If the ovaries were left in, the uterine movements were minute. Now, if Verney's *stimulus to* antidiuresis, viz., intracarotid injections of hypertonic NaCl solution, was used, then always there was increased uterine activity beginning and ending at the same time as the antidiuresis (Figure 13). The results were exactly similar whether the

contractions were large or small. In terms of injected or lobe extract, the response was equivalent to less than vasopressin and 80–100 mU oxytocin (Abrahams and d, 1954). It was from experiments such as these that the were obtained about the unequal amounts of these two nes released by a single stimulus. The behaviour of the



14 Records of spontaneous uterine contractions before and after on of experimental diabetes insipidus. A, normal, 27 May 1953

fully bore her child when she was treated with posterior lobe extracts. In dogs it is clear that the posterior lobe has a striking influence on the uterus. In a series of experiments the animals were provided with denervated carotid loops and a fistula of one uterine horn. Thus, urine flow and uterine activity could be simultaneously observed, the latter by means of a small balloon inserted through the fistula. Sometimes the ovaries were removed at the time the fistula was made. This meant

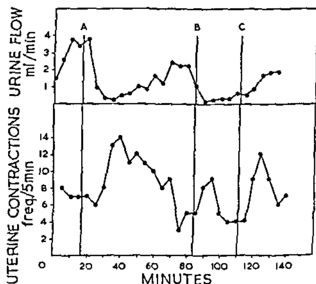


FIG 13 Effect of intracarotid injection of NaCl on urine flow and uterine motility in the conscious dog. Upper record, urine flow Lower record, frequency of uterine contraction. At zero time 350 ml water by mouth At A, 2 4 ml 1 7 M NaCl solution by intracarotid injection in 5 sec At B, 5 mU Pitressin intravenously At C, 5 mU Pitocin intravenously. (From Abrahams and Pickford, 1954)

that for about the next six weeks the uterus showed a high degree of spontaneous activity. If the ovaries were left in, the uterine movements were minute. Now, if Verney's stimulus to antidiuresis, viz., intracarotid injections of hypertonic NaCl solution, was used, then always there was increased uterine activity beginning and ending at the same time as the antidiuresis (Figure 13). The results were exactly similar whether the

REFERENCES

ABEL, J. J. (1923–24). *Harvey Society Lectures.* J. B Lippincott, Philadelphia.

ABRAHAMS, V. C Unpublished observations.

ABRAHAMS, ... *J Physiol* 126, 319

ABRAHAMS, ...

BARGMANN, ...

BARGMANN, W. and HILD, W. (1949) *Acta anat.* 8, 204.

BODIAN, D (1951). *Bull Johns Hopk Hosp.* 89, 354.

... *Brit med J* ...

... 52, 122

... 1P.

... 41.

DICKER, S. E and TYLER, C (1953). *J Physiol* 121, 206.

DUKE, H. N., PICKFORD, M. and WATT, J. A. (1950). *J. Physiol* 111, 81.

DUKE, H N, PICKFORD, M and WATT, J. A (1951). *Quart. J exp Physiol.* 36, 149.

ERÄNKO, O. and KARVONEN, M. J. (1952) *Nature,* 170, 331.

FISHER, C., INGRAM, W. R and RANSOM, S W. (1938). *Neurohormonal Control of Water Balance* Edward Bros. Inc., Michigan.

GERSCH, I (1939). *Amer J. Anat.* 64, 407.

HARRIS, G. W. (1947). *Phil. Trans.* 232, 385

HATERIUS, H O and FERGUSON, J. K. W. (1938). *Amer. J. Physiol.* 124, 314.

HILD, W. and ZETLER, G. (1951) *Experientia,* 7, 189

KAMM, O., ALDRICH, J B, GROTE, I. W, ROWE, L W. and BUGBEE, E. B. (1928) *J Amer chem Soc* 50, 573

KELLER. A. D. (1942) *Endocrinology,* 30, 408.

MARAÑON, G (1947) *Brit. med J.* ii, 769

O'CONNOR, W. J and VERNEY, E B (1942) *Quart J exp Physiol* 31, 393.

O'CONNOR, W J and VERNEY, E. B (1945). *Quart J exp Physiol* 33, 77

OLIVECRONA, H. O (1954) *Nature,* 173, 1001.

... *Klin Wschr* ...

... *hysiol* 114, 333

ROTHBALLER, A B (1953) *Anat Rec* 115, 21

SATO, G (1928) *Arch exp Path Pharmak* 131, 43

SCHARRER, E and SCHARRER, B (1945) *Physiol. Rev* 25, 171

SCHARRER, E and SCHARRER, B (1954) *Rec Progr Hormone Res* 10, 183

SMITH, P E (1932) *Amer. J. Physiol* 99, 345.

TRENDELENBURG, P. (1928) *Klin Wschr* 7, 1769

VERNEY, E B (1926) *Proc roy Soc B,* 99, 487

uterus when diabetes insipidus is induced is particularly interesting. As soon as polyuria appeared all spontaneous uterine activity ceased. During the so-called normal interphase when water exchange is minimal, due probably to an atrophic release of hormone present in the gland before operation, then spontaneous uterine activity reappeared, only to vanish finally and for good when the permanent polyuria set in (Figure 14). In our animals a moderate number of paraventricular cells remained, heavily laden with Gomori substance. Judging by this fact, and supposing that these cells can pass oxytocin direct into the blood, then a fairly large number of them seem necessary to maintain spontaneous uterine movements, as compared with the 5–10 per cent of the normal number of supraoptic cells that are sufficient for some control of water excretion. However that may be, the experiments described strongly suggest that oxytocin is, in dogs at any rate, necessary in order that the uterus may contract.

Summary

Thus, there is still a great deal that we do not know about the posterior pituitary. It is an extension of the central nervous system. The neurones of which it is a part may well make two highly active hormones which pass from the hypothalamus, where the cell bodies lie, to the lobe where they are stored and thence released when the cells take on the office of nervous tissue and send impulses down their axons. The cells may pass the hormones direct into the enormously rich capillary network by which they are surrounded. They may also have a third function and react directly to changes in osmotic pressure of the plasma as well as receive impulses from other parts of the central nervous system. At present it is not possible to say by what means more of one hormone than the other can be liberated.

Acknowledgements

I wish to express my gratitude to the editors of the following [illegible] for permission [illegible] he *Quarterly* [illegible] *lical Journal.*

XVIII

The Effects of Distension of Viscera

D. WHITTERIDGE

Activity in Afferent Nerve Fibres

Although afferent impulses from viscera have frequently been recorded previously by Talaat (1938) from the bladder and by Gernandt and Zotterman (1946) from the gut in the splanchnic nerves, there have been comparatively few records published of the activity in single nerve fibres. Here, as elsewhere, our knowledge remains vague and inexact until this has been done. Recently records of single units from the bladder have been obtained by Iggo (1955) in branches of the pelvic splanchnic nerves. He has found that these endings are stimulated by passive stretch as in filling the bladder, during active contraction with reduction in bladder volume (isotonic conditions) and especially during active contractions without change in bladder volume (isometric conditions). Many of these endings lie in the region of bladder wall near the trigone. They appear to be slowly adapting endings, but their activity is much influenced by the contraction of the surrounding smooth muscle. Its contraction always increases the afferent discharge in the endings, which presumably lie in series with the contractile elements of smooth muscle. Silence during contraction, which would be produced by elements in parallel with

the vagus below the diaphragm in the cat, efforts to lead from them were unsuccessful. This was first achieved by Paintal (1954) in the cat He picked up small fibres in the vagus in the

VERNEY, E. B. (1947). *Proc. roy. Soc. B*, **135**, 25.
DU VIGNEAUD, V., RESSLER, C., SWAN, J. M., ROBERTS, C. W., GORDON, S and KATSOYAMUS, P. G. (1954a). *J. Amer. chem. Soc.* **75**, 4879.
DU VIGNEAUD, V., GISH, D T. and KATSOYAMUS, P. G. (1954b). *J. Amer. chem. Soc.* **76**, 4751.
VOGT, M. (1953). *Brit. J. Pharmacol.* **8**, 193.
ZETLER, G. (1953). *Arch. exp. Path. Pharmak.* **218**, 116.

Reflex Activities in Spinal Lesions

Apart from these sensations, distension of viscera produces a variety of well-known reflex activities. Entrance of food into the stomach excites the gastrocolic reflex, distension of the rectum and bladder provide the 'call to defaecation' and the desire to micturate respectively, and may give rise to reflex emptying of the rectum and bladder. These mechanisms are well known and there is little new to add to the classical descriptions. There are, however, a number of vasomotor reactions, some of which have been described before, but are still sufficiently unfamiliar to be described as 'bizarre' symptoms and signs when they complicate a clinical picture. The basic reflex here is a vasoconstriction of blood vessels of the skin, muscles and perhaps the viscera, following on distension of bladder or gut (Guttmann and Whitteridge, 1947). This is most clearly seen in patients with complete spinal transections with an active but isolated spinal cord. Its existence was briefly mentioned in the masterly monograph by André-Thomas, *Le Réflexe Pilomoteur*. Most of his attention was directed, however, to pilomotor reflexes which, though of no practical consequence, have the advantage of being directly observable without special apparatus. Peripheral vasoconstriction, however, though difficult to detect clinically, is obviously a serious matter.

Evidence of cutaneous vasoconstriction can be obtained by plethysmography of the fingers and toes, and by measurement of skin temperature over the body surface. There is a decrease in calf blood flow measured plethysmographically which probably means a vasoconstriction in muscle, and the very large rise in venous pressure seen in patients with high lesions suggests that there may be active constriction of veins (Cunningham, Guttmann, Whitteridge and Wyndham, 1953). The function of this reflex is not known. It is evoked by pressures of 40–50 mm /Hg. in the lumen of the viscus, pressures which can impede the circulation in the mucosa. Widespread constriction elsewhere might improve the local circulation in the manner of a Lovèn reflex. No attempts to test this hypothesis directly have yet been made owing to the technical difficulties involved. It cannot be too strongly emphasized that these are

neck that were stimulated by the injection of phenyl diguanide and traced them down to the stomach wall and immediately adjacent mesentery. They were small fibres of conduction rate around 9 m.p.s. Most were slowly adapting with a rate of discharge related linearly to the volume of air or water used to distend the stomach. Paintal was unable to modify their activity by producing contraction of the stomach wall, but more recently Iggo has increased further their discharge by faradising the stomach wall and so producing local contraction.

These results correspond closely with those obtained in an extensive study of the afferent fibres from the reticulum in goats, by Iggo. He also found that in the quiescent stomach afferent endings were stimulated by inflation, their discharge was approximately linearly related to the volume of air introduced into the stomach, and they adapted slowly. It was comparatively easy in the goat to induce reflex contraction of the stomach as well as contraction in response to stimulation of efferent vagal fibres. In both cases the response to distension was much enhanced during contraction of the stomach wall.

Sensations in Man

It is interesting to compare these results with the classical observations on sensations evoked by gastric distension in man by Hurst (1911). He pointed out that rapid distension with very large volumes of air evoked a sensation of distension whereas slow distension as in dilatation of the stomach might not be accompanied by any sensation at all. He also remarked that doctors who came to consult him for their sensations of gastric distension usually turned out to have small contracted stomachs, and that the sensation produced by distension of a contracted stomach wall was indistinguishable from that due to rapid distension with little or no contraction.

Although these studies of afferent impulses are limited to the myelinated fibres in the vagus, and take no account of the much larger number of afferent vagal unmyelinated fibres nor of afferent fibres in the splanchnic nerves, their behaviour could provide a physiological basis for the sensations experienced in man.

duodenum (Carmichael, Doupe, Harper and MacSwiney, 1939), by a balloon introduced through an ileostomy (More, 1954), and by distension of the rectum (Carmichael, 1950). It has been seen after distension of the bladder (Adams-Ray and Norlen, 1951) most clearly when pressures of 40–50 mm./Hg. are reached during detrusor contractions (More, 1954). There is as yet little or no information on possible changes in blood flow in muscle or in viscera in man.

Distension of the bladder or rectum produces very little change in blood pressure in man (Robertson and Wolff, 1950). However, this should lead us to look for evidence of compensatory vasodilation in other vascular territories since the existence of some skin vasoconstriction in this condition seems to be established.

In studying the possible effects of distension of viscera in normal subjects, there are, of course, many sources of variation. The discharge of afferent fibres is more closely related to the pressure in the viscus than to its volume. It is well known that distension with, say, 500 ml. may cause a rise in pressure, and an irresistible desire to micturate in one subject at one time, whereas the same volume may produce very little rise in pressure in the same subject on another occasion. Studies in man without direct measurement of the pressure in the bladder are therefore useless.

The blood-pressure stabilizing mechanisms seem to have a *profound effect on these reflexes, and kidney constriction, for* example, may well only be detectable in pathological conditions when the normal controlling effect of the higher centres has been withdrawn. Lastly, of course, as is well known, the demands of temperature regulation and emotional stress can dominate the vasoconstriction mechanisms and may prevent the appearance of these reflexes.

Nevertheless in the confusion of effects of one organ on another, the 'sympathies' of the ancients, there are suggestions of true reflex viscerovisceral effects. The precipitation of attacks of angina by distension of the stomach is described by Mackenzie along with the effects of cold and emotional stress, which are known to produce vasoconstriction. The older rectal surgeons

spinal reflexes and do not depend on the production of conscious sensation, emotional change or the release of adrenaline from the adrenals.

There is a striking difference between patients with high lesions, above T_6 and those below T_6. In all patients distension of the bladder produces cutaneous constriction in the legs, but whereas the blood pressure in patients with low lesions is only slightly raised, in patients with high lesions it is raised to 250–300 mm./Hg. systolic and up to 140 mm./Hg. diastolic. Patients with low lesions show vasodilatation in their fingers, which are under the control of central blood-pressure stabilizing mechanisms. Patients with high lesions show intense vasoconstriction *in their fingers, and we suspect that there must be* considerable constriction in the splanchnic area to account for the observed rise in blood pressure with little or no change in heart output.

[illegible]

i

the cord sectioned at C_3–T_1. This was accompanied by a rise in blood pressure, was abolished by section of the splanchnic nerves, and seems to be due to renal vasoconstriction. There was also evidence of constriction in the spleen and intestines.

Reflex Activities in Intact Subjects

In the intact cat, however, Mukherjee found very little rise in blood pressure and no change in the kidney. However, after cutting the vagi and denervating the carotid sinuses and allowing time for the blood pressure to return towards normal, distending the bladder then produced a rise in blood pressure and kidney constriction. It therefore seems that in the intact animal blood-pressure regulating mechanisms prevent *all but* slight changes in blood pressure and that kidney vasoconstriction is not normally produced by this stimulus.

When we turn to intact human beings, we find the situation essentially similar. Visceral distension undoubtedly produces vasoconstriction in the skin This has been produced by distension of the oesophagus (Sturup, 1940) by a balloon in the

XIX

Fat Metabolism

A. C. FRAZER

FAT metabolism is concerned with the handling of lipid molecules by the animal body. These lipids include fatty acids and their glyceryl esters, phospholipids, cerebrosides and sterols. In the space at my disposal, I shall be able to consider one group of molecules only—the glyceryl esters. These esters may contain one, two, or three fatty acids; these may be saturated and have a chain length of between four and eighteen, or more, carbons—they usually contain an even number of carbon atoms—or they may contain unsaturated fatty acids with one or more double bonds. Certain poly-unsaturated fatty acids cannot be synthesized in the body and they are, therefore, dietary essentials. One other molecule we must consider is lecithin, which is a glyceryl ester containing two fatty acids and a phosphoryl choline group in place of the third fatty acid.

Terroine (1919) introduced an important concept into the consideration of fat metabolism when he pointed out that the body lipids could be broadly divided into two groups—'l'élément constant', consisting of structural lipids that did not enter into immediate metabolism, and 'l'élément variable', which included the lipids that were taking an active part in energy production Isotope studies have, of course, shown that even the structural molecules in the tissues are being continually turned over; nevertheless, it is important to appreciate that the lipids in any tissue may fulfil two different functions—one concerned with the continuing structure of the cell and the other with the provision of fuel for energy production. The glyceryl esters, which we shall now consider, are mainly concerned with this latter function—energy production.

used to teach that piles were commonly accompanied by indigestion. The relation between distension of the gall bladder and coronary disease has long been puzzling. According to Machella (1949), the symptoms of the 'dumping' syndrome can be reproduced by distending the jejunum. This has been confirmed by Glazebrook and Welbourn (1952) who add that during spontaneous attacks the jejunum seems to be contracted. If the relevant endings are in series with the smooth muscle, as Iggo's work suggests, the 'contracted' jejunum could give rise to much afferent discharge. These suggestions are difficult to investigate and easy to dismiss with a facile reference to psychosomatic mechanisms. I wish to suggest that on the contrary they may be spinal reflex mechanisms best investigated perhaps in those paraplegics who now survive in large numbers and in a state of remarkable physical fitness.

REFERENCES

ADAMS-RAY, J. and NORLEN, G. (1951) *Acta physiol. scand.* 23, 95
ANDRÉ-THOMAS (1921). *Le Réflexe Pilomoteur.* Masson, Paris
CARMICHAEL, E. A. (1950). *Brit. med Bull.* 6, 351.
CARMICHAEL, E A., DOUPE, J., HARPER, A A. and MACSWINEY, B. A. (1939) *J. Physiol.* 95, 276
CUNNINGHAM, D. J. C, GUTTMANN, L., WHITTERIDGE, D. and WYNDHAM, C. H (1953). *J. Physiol.* 121, 581.
GERNANDT, B. AND ZOTTERMAN, Y. (1946). *Acta physiol. scand.* 12, 56.
GLAZEBROOK, A. J. and WELBOURN, R. B (1952). *Brit. J Surg.* 40, 111.
GUTTMANN, L. and WHITTERIDGE, D (1947). *Brain,* 70, 361.
HURST, A. F. (1911). *The Sensibility of the Alimentary Canal.* Oxford Medical Publications.
IGGO, A. (1955) *J Physiol* 128, 593.
MACHELLA, T. E (1949) *Ann. Surg.* 130, 145.
MORE, B. (1954). *Unpublished observations.*
MUKHERJEE, S R. (1955). Ph D. thesis, Edinburgh University.
PAINTAL, A. S. (1954) *J Physiol.* 126, 255.
ROBERTSON, H A. and WOLFF, H G. (1950). *Arch. Neurol. Psychiat (Chicago),* 63, 52
[illegible] *Copen-*
hagen.

There is considerable evidence indicating that, in the course of this metabolism, it is not necessary for the fat to be brought into aqueous solution as fatty acid or soap for absorption to occur (Frazer, 1946). Studies with isotope-labelled materials confirmed that complete hydrolysis to fatty acid and glycerol is not an obligatory step in fat absorption (Favarger *et al.*, 1951; Karnovsky and Gidez, 1951; Reiser *et al.*, 1952).

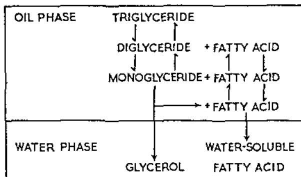


FIG 1 *Hydrolysis and resynthesis of glycerides*

Fine Emulsification

The fatty material in the diet may be unemulsified, but both in the intestinal lumen and in the chyle the fat is dispersed as an oil-in-water emulsion with an average particle size of 0·5 μ or less. The emulsification of the glyceride fraction in the small intestinal lumen is brought about by the triple system of fatty acids/lower glycerides/bile salts (Frazer, Schulman and Stewart, 1944). It is thus dependent upon the presence of both pancreatic lipase and bile, as demonstrated by Claude Bernard a hundred years ago. It has been thought that this fine emulsification is important for rapid and complete lipolysis It can be shown, however, that rapid and complete hydrolysis need not occur Is there, therefore, any other significance in the fine emulsification of the water-insoluble fraction? To answer this question, we must digress for a moment to consider the intestinal absorption of certain other substances, such as paraffin and high melting-point esters

Intestinal Absorption of Glycerides

The main differences in the fatty materials found in the diet, the intestinal lumen and the chyle are shown in Table 1. Critical examination of these changes indicates the more important points in the fat-absorption mechanism.

Table 1. Fatty materials in the diet, the intestinal lumen and the chyle, during glyceride absorption

	Diet	Intestinal Lumen	Chyle
Type of Glyceride	Triglycerides	Triglycerides Diglycerides Monoglycerides	Triglycerides
State	Usually unemulsified	Finely emulsified	Finely emulsified
Phospholipid	Variable	Phospholipid formed	6–10% Phospholipid present
Fatty acids	Long Chain Short Chain (<C10)	Long Chain Short Chain (<C10)	Long Chain

Hydrolysis and Resynthesis of Glycerides

The series of reactions illustrated in Figure 1 are catalysed by lipase in the intestinal lumen. Under the conditions that prevail, hydrolysis is usually partial and the intestinal contents consist of triglycerides, diglycerides, monoglycerides and free fatty acids. The amount of glycerol set free may be quite small. (Frazer and Sammons, 1945; Desnuelle, Naudet and Constantin, 1950). Since the dietary glycerides are mainly triglycerides, *it is obvious that the direction of this reaction* in the intestinal lumen is from above downwards. Examination of the chyle reveals that the glycerides present there are predominantly triglycerides. Thus, during the passage of the glyceride mixture through the intestinal wall, the equilibrium *of this reaction has shifted from upwards (Figure 1).*

Although we start with triglycerides in the diet and end with triglycerides in the chyle, these molecules may have undergone metabolic changes in their passage through the intestinal wall.

There is considerable evidence indicating that, in the course of this metabolism, it is not necessary for the fat to be brought into aqueous solution as fatty acid or soap for absorption to occur (Frazer, 1946). Studies with isotope-labelled materials confirmed that *complete hydrolysis* to fatty acid and glycerol is not an obligatory step in fat absorption (Favarger *et al.*, 1951; Karnovsky and Gidez, 1951; Reiser *et al.*, 1952).

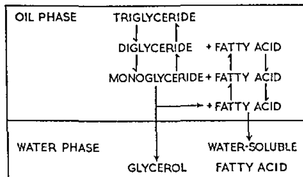


FIG. 1 Hydrolysis and resynthesis of glycerides

Fine Emulsification

The fatty material in the diet may be unemulsified, but both in the intestinal lumen and in the chyle the fat is dispersed as an oil-in-water emulsion with an average particle size of 0·5 μ or less The emulsification of the glyceride fraction in the small intestinal lumen is brought about by the triple system of fatty acids/lower glycerides/bile salts (Frazer, Schulman and Stewart, 1944). It is thus dependent upon the presence of both pancreatic lipase and bile, as demonstrated by Claude Bernard a hundred years ago It has been thought that this fine emulsification is important for rapid and complete lipolysis It can be shown, however, that rapid and complete hydrolysis need not occur. Is there, therefore, any other significance in the fine emulsification of the *water-insoluble fraction*? To answer this question, we must digress for a moment to consider the intestinal absorption of certain other substances, such as paraffin and high melting-point esters.

It is well known that liquid paraffin B.P. is not absorbed to any appreciable extent if it is fed alone. Several workers have shown that paraffin may be absorbed in small amounts if it is fed in the diet (Channon and Collinson, 1929; Stetten, 1943), but the mechanism of absorption was not studied. We found that paraffin finely dispersed to a particle size of less than 0·5 μ disappeared from the intestinal lumen and was taken up into the intestinal cells (Frazer, Schulman and Stewart, 1944). We also showed that liquid paraffin was well absorbed from mixtures of paraffin and olive oil, but not from mixtures of paraffin and tributyrin—the former was finely emulsified in the intestinal lumen, while the latter was not. The paraffin was not destroyed in the intestinal lumen and the absorbed paraffin, recovered from the intestinal cells, chyle, liver and carcass, was identified by elementary analysis. However, the quantity of paraffin recovered was only a small proportion of the quantity that had disappeared from the intestinal lumen (Daniel, Frazer, French and Sammons, 1953).

A second series of studies is concerned with the absorption of a tartaric acid ester of stearyl alcohol. This had a melting-point of 63° C., it was insoluble in water, but soluble in olive oil; it was not hydrolysed by pancreatic lipase. When fed as a suspension in water it was not absorbed, but if it was fed in olive oil, 60 per cent absorption occurred (Frazer, Pover and Sammons, 1953).

The third observation that has some bearing on this problem was made in children with no pancreatic lipase. The absence of lipase was demonstrated by intestinal intubation. The small intestine was normal in its absorptive capacity for glucose, or other water-soluble substances, and it appeared normal radiographically. The fat was not emulsified in the intestinal lumen, there was a severe fat-absorption defect, and no significant increase in the number of chylomicrons occurred in the systemic blood for the six hours following fat feeding. If finely emulsified fat was administered intraduodenally to such patients, there was a marked increase in the rate of absorption of the glyceride fraction and in the number of chylomicrons in the systemic blood during the five hours following the administration of the

fat. The particle size of the emulsions that caused these effects was critical; emulsions of the order of 1–2 μ or more particle size were not effective; emulsions with particles averaging less than 0·5 μ diameter caused the response (Anderson, Daniel, Frazer, French and Sammons, 1952).

It is concluded from these various studies that fine emulsification of fat in the intestinal lumen to a particle size of less than 0·5 μ is an important step in the removal of the water-insoluble fraction from the intestinal lumen that is independent of any facilitating action on lipolysis.

I want to suggest a possible reason why this fine emulsification—or the physical chemical changes that accompany it—may be important in the absorption of the water-insoluble residue. In 1942 J. R. Baker described fine canals in the outer border of the small intestinal cell. These were seen in both fixed and unfixed preparations and their size was of the order of 0·5 μ. Some ten years later Baker (1951) also described similar structures in the outer border of the intestinal cell, which appeared as lipid-staining spindles during fat absorption; they were not seen during absorption of other food materials. These spindles were similar in size, shape and distribution to the canals originally described. We and others have repeatedly confirmed Baker's observations (Hewitt, 1954). There is no doubt that fat is distributed in the outer border of the intestinal cell during fat absorption in particles about 0·5 μ in diameter. This distribution brings the fatty material into close contact with the surface of the small intestinal cell and this contact is not made by particles bigger than about 0·5 μ diameter. Electron microscope studies have resulted in a further analysis of the structure of the brush border, which has been shown to have a fine fibrillar appearance (R. F. Baker and Grainger, 1950). This ultra structure could, however, co-exist with the canal structure described by J. R. Baker. The canals have not been shown up convincingly by electron-microscope studies, but one may doubt whether sections 0·1 μ thick are a suitable way of demonstrating canals 0·5 μ across. For the purposes of this general argument, however, it is unimportant for the canals to be permanent fixed structures.

The question has been raised whether the same glyceride particle observed in the lumen, passes through the cell, into the chyle and so into the blood. It is obvious from what has already been said that the constituent molecules of the particle may undergo considerable modification while passing through the intestinal wall. The crux of this question, however, seems to be whether there is some sort of molecular transport mechanism in the intestinal cell. This cannot be excluded on present evidence. If a molecular transport mechanism exists, it does not appear to function simply on a basis of molecular size or water solubility; glycerides, liquid paraffin, long-chain alcohols and certain sterols pass through. Both the glycerides and liquid paraffin undergo modification in the course of their passage through the cell—it is not impossible that some molecular modification may determine entry into the intestinal cell.

Phospholipid Formation

There is no doubt that lecithin is formed in the intestinal cells. This was demonstrated by Artom and Peretti (1935) and Sinclair and Smith (1937), who traced various lipid components from the diet into these phospholipids. However, from isotope studies on phosphorus turnover in the intestine during fat absorption, Zilversmit, Chaikoff and Entenman (1948) concluded that phospholipid was not an obligatory intermediate in the absorption of glycerides. Lecithin, choline and glycerophosphate added to fat cause a significant increase in the rate of glyceride absorption (Frazer, Pover and Sammons, 1953).

Several workers have reported the presence of lecithinases in the intestine (Kahane and Levy, 1944; Schmidt, Hershman and Thannhauser, 1945; Le Breton and Pantaléon, 1947). Distinction has not always been made, however, between endocellular and extracellular enzymes. We were unable to demonstrate significant lecithin-splitting activity in the intestinal contents of the rat, or human subjects, but found that an extract of rat intestinal mucus membrane had marked lecithinase activity (Frazer, Sagrott and Sammons, 1949). This is in agreement with the observations of Kahane and Levy, and

Schmidt *et al.* Several enzymes are available that affect the lecithinase molecule; glyceryl-phosphoryl-choline may be formed and this may take up fatty acids to form lecithin. Whether these, or other phosphorylating reactions, play any part in triglyceride resynthesis is not yet known. Lecithin may, of course, occur in the diet and it is present in the bile, but it does not seem to play an important part in the stabilization of the intraluminar oil-in-water emulsion. In the blood and in the chyle, on the other hand, lecithin is usually essential to the stability of the fat particles, the addition of lecithinase-D results in rapid clumping and creaming (Frazer, 1947). These lecithin-stabilized fat particles are associated with the globulin fraction and remain discrete in the presence of plasma proteins, in contrast to negatively charged, soap-stabilized fat particles, which flocculate in the presence of plasma proteins at pH 7·4 (Elkes, Frazer, Schulman and Stewart, 1945). In studies on a human subject with a thoracic duct fistula, we observed fat particles that appeared to be stabilized by albumin rather than globulin; the isoelectric point of the particles was at pH 4 6 instead of 5 6 and the flocculation reaction corresponded to that of albumin; the lymph contained a high A/G ratio. These fat particles did not flocculate in the presence of lecithinase-D (Frazer, 1949) A similar observation has since been reported by Laurell (1954). Whether these apparent differences of surface structure of particulate glycerides have any biological significance is not yet established; the persistence of such differences might be expected to modify the subsequent behaviour of the particles.

Changes in Fatty Acid Composition: Partition

In a two-phase oil/water system, the various molecules present will become distributed between the oil and the water phases according to their respective partition co-efficients. This must happen in the intestine in the course of absorption. The products of lipolysis may leave the oil and pass into the water phase. This occurs most readily with glycerol and the shorter-chain fatty acids.

The disappearance of the short-chain fatty acids from the oil phase recovered from the chyle has been known for many years

(Davis, 1930; Hughes and Wimmer, 1935). Other differences between the absorption of long- and short-chain glycerides have been noted, and I suggested the possible significance of these differences in terms of partition in 1946. On this basis, the molecules follow either the oil or water phase; the particulate oil phase seems to pass almost exclusively in the chyle, while the water phase is distributed between the chyle and the portal blood. Since the portal flow is large as compared with chyle flow, the water-soluble group appear to pass preferentially by the portal route. Chaikoff and his co-workers (1950–2), using isotope-labelled fatty acids, have put forward convincing evidence that fatty acids with a chain length of ten carbons or less do not appear in the chyle; they are present in larger amounts in the portal blood than the longer-chain fats.

Although removal from the oil phase is a question of simple partition, there may be other factors involved in the failure of the short-chain fatty acids to come back into the glyceride cycle. While this may be partly due to physical separation, it may also be related to the actual enzymic reactions involved in these syntheses—differentiation between the fatty acids with more or less than ten carbons is also observed in fatty acid oxidation. Some utilization of these shorter-chain fatty acids by the intestinal cells cannot be ruled out. The present evidence would suggest, however, that they were not converted into longer-chain fatty acids in the intestinal cell of the rat to any appreciable extent.

To summarize the information on the intestinal absorption of glycerides, we may conclude that hydrolysis may be partial and not complete; fine emulsification of the glyceride residue is an important step in its absorption; this may be necessary to bring the fatty material into close contact with the intestinal cell surface; considerable metabolic activity occurs in the intestinal cell and lipid molecules may undergo modification during absorption (the possibility that certain molecular modifications are necessary for entry into the intestinal cell cannot be ruled out); phospholipid formation occurs in the intestinal cell and appears to be closely related to glyceride absorption; the lipid molecules are distributed between the oil and the water phase,

according to their partition co-efficients; the oil phase passes almost exclusively in the chyle, while the water phase is distributed both ways; since portal flow is much greater than chyle flow, water-soluble materials pass mainly in the portal blood; fatty acids with less than ten carbons do not pass in the chyle and are not concerned in glyceride resynthesis; they pass in greater amount in the portal blood, but other factors may play a part in their disappearance from the glyceride cycle.

Glycerides in the Blood

Let us now turn our attention to the glycerides in blood. The distribution is essentially between the plasma and the white blood corpuscles—the red blood cells contain practically no glycerides and the phospholipids present are structural components. Singularly little is known of the significance of the fat content of white blood cells, although it may be quite considerable (Burt and Rossiter, 1950).

Fasting Blood Plasma

Fasting plasma is a clear fluid. A standard film is found to contain few visible particles when examined under dark ground illumination. The amount of glyceryl esters present is of the order of 100 mg. per 100 ml. Phospholipid amounts to about 150 mg. per 100 ml. in human plasma, 70–80 per cent of which is of the lecithin type.

It is now well known that the blood lipids are closely associated with plasma proteins in the form of lipoproteins, which form particles or macromolecules. By various methods it is possible to separate and characterize some of these classes of lipoproteins. By the use of alcohol precipitation methods, Cohn and his group (Gurd, Oncley, Edsall and Cohn, 1949) were able to prepare two distinct lipoproteins—a β-lipoprotein that accounted for 75 per cent of the lipid in plasma and an α-lipoprotein. The β-lipoprotein was a spherical particle about 180 Å in diameter, with a molecular weight of about 1 3 million, while the α-lipoprotein was cigar-shaped, being some 300 Å long and 50 Å wide, and having a molecular weight of 200,000. Lipoproteins can also be studied by ultracentrifugation and

characterized by their flotation properties, as indicated in Table 2. Normal fasting plasma contains moderate amounts of Sf 4 and Sf 6 lipoproteins and minimal quantities of all the higher groups. Age and disease are associated with an increase of the Sf 10–30 lipoproteins and this has been shown by Gofman and his group (1950) to be significantly correlated with the development of atherosclerosis. We must, however, consider the

TABLE 2. Relationships of Glycerides and Lipoproteins

Sf	Quantity in Normal fasting Serum	Quantity in Hyperlipaemic Serum	Glyceride Content
4–6	Moderate	No change	Nil
10–20	Minimal	No change	Nil
20–100	Minimal	Increased	Present
40,000 (chylomicrons)	Minimal	Increased	80% or more

relationship between these lipoproteins and glycerides. There are practically no glycerides in the Sf 4–6 lipoproteins and only minimal quantities in any of the fractions up to Sf 20. From this point onwards, glyceryl esters form an increasingly larger proportion of the particle. It is not surprising, therefore, to find that a diurnal variation was demonstrated in the case of the Sf 20–100 lipoproteins, but not for the Sf 10–20 group (Chandler *et al.*, 1953).

Hyperlipaemic Plasma

Under certain circumstances there is a marked increase in the glyceride content of the blood plasma and this is accompanied by concomitant increase of phospholipid and cholesterol. The increase of glyceride is accompanied by the development of turbidity of the plasma and a marked increase in the number of particles observed under dark ground illumination in a standard film. These particles consist mainly of glyceryl esters containing long-chain fatty acids. The particles migrate with the globulin fraction of the plasma protein and give the flocculation characteristics of globulin; the particles clump together and cream if treated with lecithinase-D. It has, therefore, been

concluded that, as in the case of the fat particles in chyle, the lecithin and globulin form the basic stabilizing film.

The largest and brightest particles are of the order of 0 5 μ in diameter. There is a gradation downwards to innumerable smaller and duller particles. The smallest dull particle measured by us had a diameter estimated as 35 mμ (Elkes, Frazer and Stewart, 1939). This size is similar to that of the lipoprotein particles of the Sf 20 series. It seems that the glyceride in the hyperlipaemic plasma is dispersed into particles of different sizes—the smallest glyceride-containing particle being of the order of about 35 mμ. The quantity of glyceride in hyperlipaemic plasma is commonly of the order of 600 mg. per 100 ml. of blood. Under certain circumstances it may be considerably greater than this—in essential hyperlipaemia the glyceride levels may exceed 10 g. per 100 ml.

Alimentary Hyperlipaemia

The commonest form of hyperlipaemia and the one that has been most studied is that associated with the absorption of glycerides. We have already considered how particulate glyceride passes into the chyle after absorption. This fat-laden chyle is discharged into the systemic blood from the thoracic duct. All the particulate fat in the chyle is not necessarily derived from fatty material that has just been absorbed—if labelled fats are used, it will be found that the labelled glyceride in the thoracic duct chyle is considerably diluted by unlabelled glycerides. The particulate fat is rapidly mixed with the circulating plasma, giving rise to the classical hyperlipaemia. The course of the hyperlipaemia may be followed either by serial analyses, turbidity measurements, or counts of visible particles under standard conditions—all three methods give substantially the same results. After the ingestion of about 30 g. of fat, the hyperlipaemia [illegible] [illegible] [illegible] returns to the fasting state by about 5 hours. The hyperlipaemic changes are dependent upon the relative rates of income and removal of the particulate glyceride. The former depends on the

rate of discharge of fat-laden chyle from the thoracic duct, which, in turn, depends on the rate of absorption from the intestine. The rate of removal of the particulate glyceride from the blood can be affected by heparin, so that the alimentary hyperlipaemia can be apparently completely suppressed (Hahn, 1943; Weld, 1944). Before going further, we must consider this effect of heparin on particulate blood fat in greater detail.

The Effect of Heparin on Particulate Blood Fat

The administration of heparin to a fat-absorbing rat or dog resulted in disappearance of the normal systemic hyperlipaemia. This effect was counteracted by an anti-heparin, such as protamine, and appeared to be due either to finer dispersion of the particulate fat in the plasma, or more rapid removal from the blood, or both (Anderson and Fawcett, 1950; Swank and Wilmot, 1951, Brown, 1952). The addition of heparin to hyperlipaemic plasma did not cause clearing *in vitro*, but the phenomenon did occur if plasma or serum from a recently heparinized individual was used. The active component in this serum was termed the 'clearing factor' (Anfinsen *et al.*, 1952; French, Robinson and Florey, 1953). The 'clearing factor' was thermolabile and the optimum pH for its action appeared to be 7·4–7·6 (Robinson, Jeffries and French, 1954). The action was associated with enzymic breakdown of the plasma lipids—it seemed likely that the 'clearing factor' was one or more lipoclastic enzymes which were either liberated or activated by the administration of heparin (Shore, Nichols and Freeman, 1950; French *et al.*, 1953). The 'clearing factor' caused a decrease of the lipoproteins of the Sf 20–100 group, which appeared to be converted to lipoproteins of lower mobility by removal of glyceride (Jones *et al.*, 1951). The fatty acid liberated could be measured and this was normally taken up by the albumin fraction of the plasma protein—the amount of albumin available was a limiting factor in the clearing reaction *in vitro* (Robinson and French, 1953). The heparin 'clearing factor' also caused clumping of chylomicrons (Swank, 1951). These various interrelationships are illustrated diagrammatically in Figure 2

To what extent do these interesting observations play any part in the handling of particulate glyceride in the normal animal? The various components concerned in the heparin clearing effect are present in the normal animal body. Brown (1952) showed that the administration of protamine resulted in a significantly greater hyperlipaemia in the normal fat-absorbing rat. Jeffries (1954) demonstrated the formation of

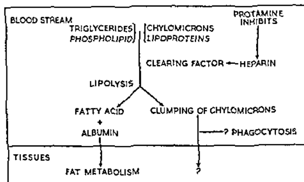


FIG. 2 The effects of the heparin 'clearing factor' on blood glycerides.

a clearing factor with similar characteristics to the heparin clearing factor during fat absorption in rats. The development of clearing activity appeared to be related to the presence of particulate fat in the blood. It is, therefore, a definite possibility that the production of a clearing factor similar or identical with the heparin clearing factor may be part of the normal physiological mechanism of disposal of blood glycerides

The rate of removal of glyceride fat from the blood stream is sometimes grossly abnormal, particularly in essential hyperlipaemia. In these patients the blood fat builds up to extremely high levels, often exceeding 10 g of glyceryl esters per 100 ml. of blood. Using labelled fats, it can be shown that the rate of removal of the glyceride from the blood stream is excessively slow (Stanley and Thannhauser, 1949). If glycerides are restricted in the diet, the blood-fat levels gradually fall. The high blood-glyceride level may not cause any symptoms and the condition is often discovered accidentally. Heparin does not cause any dramatic reduction of the blood-fat level in these

patients. Further studies on patients of this type, and other types of hyperlipaemia, might throw more light on the interrelationships of these various factors.

Glycerides in the Liver and Tissues

We must now consider what eventually happens to glycerides in the tissues. It is generally accepted that a fair proportion of the glycerides circulating in the systemic blood after absorption from the intestine are deposited in the fat depots. The other likely fate is oxidation.

Fat Depots

Labelled fats have been traced to the depots and the half life of these fat stores has been estimated as 6–8 days in the rat (Schoenheimer and Rittenberg, 1936). In animals that consume fairly large quantities of fat, the composition of the fat depots closely resembles the fat transported in the chyle; alterations in dietary fat are reflected in the composition of the fat depots. In animals that eat little fat and synthesize much from carbohydrate, no correlation is found between dietary and depot fat. It seems clear, therefore, that some of the particulate glyceride fat observed in alimentary hyperlipaemia in omnivorous or carnivorous animals will be deposited in adipose tissue cells. Must this glyceride be hydrolysed to fatty acid before it can be transferred from the blood into the fat depots?

It has been stated recently that many tissues incorporate labelled fatty acid into glycerides, but this was not accomplished by adipose tissue cells (Jederkin and Weinhouse, 1954). If this is true, glycerides must be transferred from the blood to the fat depot cells as such. On the other hand, there is fairly extensive evidence of fat formation from carbohydrate in adipose tissue cells. It is possible, however, that more than one type of cell is involved in these reactions. At present we must admit that we do not know how fat is deposited in fat depot cells. One possibility is that the clumping of the chylomicrons already described may result in their temporary arrest in the capillaries and subsequent phagocytosis.

The mobilization of glyceride from the adipose tissue cells is

equally obscure. There is some indication that this can be affected by pituitary, adrenal and other hormones. The integrity of the normal nerve supply to adipose tissue is also supposed to affect mobilization.

Fat Oxidation

The mechanism of fat oxidation is now fairly well understood and an excellent review of this subject has been published recently by Green (1954). Although other forms of oxidation may occur, the main pathway appears to be by β-oxidation of activated fatty acids which are combined with co-enzyme A. The 2-carbon fragments formed are completely oxidized in the citric acid cycle. The final 2-carbon fragment requires special treatment. If the citric acid cycle is not operating at full capacity, a greater proportion of the acetyl-CoA becomes directed towards acetoacetate, with consequent ketosis. This is the situation in the diabetic liver.

It was thought at one time that fats had to undergo preliminary desaturation in the liver before they could be oxidized. This concept partly arose from the fact that liver fats have a much higher iodine value than depot fats. This, however, is mainly due to the high proportion of poly-unsaturated fatty acids, such as arachidonic acid, in the liver phospholipids—these fatty acids are well conserved, however, and do not undergo oxidative catabolism. It is not necessary for fats to pass through the liver before being oxidized in the extra-hepatic tissues, nor need preliminary desaturation take place. The main part of the fatty material with which we are concerned is present in the blood as glyceryl esters and lipolysis is necessary before β-oxidation can occur. The heparin clearing factor would seem to provide a suitable mechanism for hydrolysis in the blood stream and it may well be that the fatty acid/albumin complex is the normal pathway leading to β-oxidation in the tissues. There are reasons, however, for thinking that this is not quite the full story—the most convincing being the abundant evidence indicating that phospholipid formation, especially in the liver, plays an important part in these final stages of fat metabolism. We may, perhaps, conclude our consideration of

fat metabolism by a brief review of this complicated aspect of our problem.

The Role of the Liver in Fat Metabolism

No one doubts that the liver plays an important part in glyceride metabolism. The available facts, however, present a strangely confusing picture. The liver appears to be an active centre for fat oxidation—this entails β-oxidation of activated fatty acids, as already described. If acetoacetate is formed in substantial quantities, the liver is unable to deal with it and it passes into the blood to be dealt with by the extrahepatic tissues, the surplus spilling over into the urine. Fatty acids may reach the liver in free form via the portal blood—this is particularly the case with the shorter-chain fatty acids, all of which are readily oxidized. The longer-chain fatty acids may also be passed to the liver as the fatty acid/albumin complex built up by the heparin clearing factor.

It is also well established that the liver can synthesize phospholipids. It may, perhaps, be wise to give some thought to the possibility, originally suggested by Sinclair (1935) that two main types of lecithin are formed in the liver. One type, containing arachidonic acid, is situated in the mitochondria, where it is closely associated with the functional activity of certain enzymes, such as succinic oxidase. This type of lecithin seems to be well conserved—at least, the essential fatty acid portion is retained or re-utilized, so that the effects of dietary deficiency of essential fatty acids may be delayed for a considerable period in adult animals The other type of lecithin does not contain such highly unsaturated fatty acids and may be more closely related to the day-to-day turnover of fatty acids and glycerides It is likely that the turnover of both types of lecithin will be affected by choline deficiency, but the effects of this diminished turnover may differ The function of phospholipid synthesis in the liver is obscure. It has been shown that it determines the level and the rate of turnover of lecithin in the plasma, but the evidence does not support the view that the lecithin is taken up or utilized by the extrahepatic tissues to any significant extent. The only conclusion that seems reasonable is that the lecithin formation in the liver is concerned with the

maintenance of adequate levels of lecithin in the mitochondria and in the plasma lipoproteins.

Even more puzzling is the effect of choline deficiency on liver fat—the characteristic feature of which is accumulation of glyceryl esters in the liver cells (Best and Lucas, 1943). There is overwhelming evidence to show that the basic effect of the choline deficiency—and also of closely related faults in transmethylation, which have a similar effect on liver lipid—is interference with lecithin synthesis (Perlman, Stillman and Chaikoff, 1940; Stetten, 1941; Welch and Landau, 1942; McArthur, Lucas and Best, 1947). Why should failure of lecithin synthesis lead to the accumulation of glyceryl esters in the liver? The fatty acids in the glyceryl esters are derived at least in part from the fat-depot glycerides and are affected by dietary glycerides (Channon *et al.*, 1942). Are glycerides normally transferred from the depots, or the absorbed dietary fat, to the liver as glyceryl esters and passed into the liver cells as such? If so, what of the heparin clearing mechanism and its hydrolyzing enzymes? Still more peculiar is the fact that there is no lipase to be found in the liver that is effective against long-chain glycerides. It is a problem for the liver to handle these particular lipids and, as already reported, the liver appears to inactivate blood lipase. Could triglyceride be converted into lecithin as part of the catabolic pathway? There is some evidence from isotope studies on intermediates in the liver that lecithin may be broken down to glyceryl-phosphorylcholine and fatty acids (Dawson, 1955). A further explanation might be that the diminished phospholipid level results in decreased utilization of fatty acids and that the glyceride represents an alternative end-product to lecithin. Finally, glyceryl esters may not be deposited in the liver cells under normal circumstances and this effect may be a further result of choline deficiency. I am afraid we cannot decide between these various possibilities on present evidence; the final answer may involve more than one of them

SUMMARY

To summarize the main facts about the transport and utilization of glycerides, we may conclude that glycerides are transported

in particulate form in the blood—the size of the particles containing glycerides ranges from about 0·5 μ down to the region of 30 mμ; increase of particulate glycerides in the blood results from the ingestion of glycerides, or the mobilization of stored glycerides; the mobilization of endogenous glycerides can be stimulated by a number of hormones and also appears to be related to nervous factors; the removal of glyceride from the blood stream is affected by heparin, which appears to cause a clearing factor to become effective; this clearing factor consists of one or more lipolytic enzymes—the released fatty acid is taken up by albumin; the clearing factor also causes clumping of chylomicrons; a clearing factor of this type may be the physiological mechanism by which glyceride is removed from the blood stream; glyceryl esters are deposited in the fat depots —this may account for as much as 50 per cent of the absorbed glyceride; it is claimed that labelled fatty acid is not incorporated into glycerides in adipose tissue cells, in which case the glyceride esters must have been deposited as such; the glycerides in the fat depots are being continually turned over—the half life has been estimated as 6–8 days in the rat; the other fate of blood glycerides is oxidation; β-oxidation of activated fatty acids, combined with co-enzyme A, appears to be the main mechanism for fat oxidation; it can occur in many tissues and it seems likely that the fatty acid/albumin complex may be used in this way; in addition to participation in fatty acid oxidation, the liver is also concerned in active lecithin synthesis; the lecithin may be of two main types—one associated with mitochondrial structure and containing highly unsaturated arachidonic acid; the other a more labile metabolic lecithin containing the less unsaturated fatty acids found in the circulating glycerides; choline deficiency causes gross interference with phospholipid turnover; this causes the accumulation of glyceryl esters in the liver—the precise reason for this effect remains obscure.

REFERENCES

ANDERSON, C , DANIEL, J W., FRAZER, A. C , FRENCH, J. M. and SAMMONS,

d. N.Y 74, 768.

ce, 115, 583.

BAKER, R F. and GRAINGER, B. (1950). *Anat. Rec* **107**, 423

BERNARD, C (1856). *Mémoire sur le pancréas et sur la rôle du suc pancréatique dans les phénomènes digestifs, particulièrement dans la digestion des matières grasses neutres* Baillière, Paris

BEST, C. H. and LUCAS, C. C. (1943). *Vitamins and Hormones*, **1**, 1.

BLOOM, B , CHAIKOFF, I. L., REINHARDT, W. O , ENTENMAN, C. and DAUBEN, W. G. (1950) *J. biol Chem.* **184**, 1.

BLOOM, B , CHAIKOFF, I. L. and REINHARDT, W. O. (1951). *Amer. J. Physiol.* **166**, 451.

BLOOM, B., CHAIKOFF, I. L., REINHARDT, W O and DAUBEN, W. G. (1951). *J. biol. Chem* **189**, 261.

BROWN, W. D. (1952) *Quart. J. exp. Physiol* **37**, 75

BURT, N. S. and ROSSITER, R. J. (1950) *Biochem. J.* **46**, 569

CHAIKOFF, I. L., BLOOM, B , STEVENS, B P., REINHARDT, W. O. and DAUBEN, W. G. (1951). *J. biol. Chem* **190**, 431.

CHANDLER, H. L , LOWRY, E. V , POTEE, K. G. and MANN, G. V (1953). *Circulation*, **8**, 723.

CHANNON, H. J. and COLLINSON, G. A (1929). *Biochem. J* **23**, 676.

CHANNON, H J , HANSON, S. W. F. and LOIZIDES, P. A (1942) *Biochem J.* **36**, 214.

DANIEL, J W., FRAZER, A C , FRENCH, J M and SAMMONS, H G (1953). *J Physiol* **114**, 26 P.

DAVIS, R E. (1930). *J. biol. Chem* **88**, 67

DAWSON, R. M C. (1955) *Biochem. J* **59**, 5

DESNUELLE, P , NAUDET, M and CONSTANTIN, M J (1950). *Biochim biophys. Acta*, **5**, 561.

DESNUELLE, P , NAUDET, M. and CONSTANTIN, M J. (1951) *Biochim. biophys. Acta*, **7**, 251.

ELKES, J J., FRAZER, A C and STEWART, H C (1939). *J Physiol* **95**, 68

ELKES, J J., FRAZER, A C , SCHULMAN, J H and STEWART, H C (1945). *Proc. roy. Soc* , A, **184**, 102.

FAVARGER, P., COLLET, R. and CHERBULIEZ, E (1951). *Helv chim. Acta*, **34**, 1641.

FRAZER, A C (1946) *Physiol. Rev.* **26**, 103.

FRAZER, A. C. (1947) *Surface Chemistry*. Butterworth, London

FRAZER, A. C (1949). *Discussions of the Faraday Society*, **6**, 81.

FRAZER, A. C. and SAMMONS, H. G. (1945). *Biochem J.* 39, 122.

FRAZER, A. C., POVER, W. F. R. and SAMMONS, H. G. (1953). Internat Conference on Biochemical Problems of Lipids. Paleis der Academiën, Brussels.

FRAZER, A. C., SAGROTT, P. E. and SAMMONS, H. G (1949). *Proc. internat. Congr. Biochem.* 1, 596.

FRAZER, A. C., SCHULMAN, J. H. and STEWART, H. C. (1944). *J. Physiol.* 103, 306.

FRENCH, J. E., ROBINSON, D. S. and FLOREY, H. W. (1953). *Quart. J exp. Physiol.* 38, 101.

GOFMAN, J. W., JONES, H. B., LINDGREN, F. T., LYON, T. P., ELLIOTT, H A. and STRISOWER, B. (1950). *Circulation,* 2, 161.

GREEN, D. E. (1954). *Biol. Rev.* 29, 330.

GURD, F. R. N., ONCLEY, J. L., EDSALL, J. T. and COHN, E J. (1949) *Discussions of the Faraday Society* 6, 70.

HAHN, P. F. (1943). *Science,* 98, 19

HEWITT, W. (1954) *Quart. J. micr. Sci* 95, 153.

HUGHES, R. H. and WIMMER, E J. (1935). *J. biol. Chem.* 108, 141.

JEDERKIN, L. H. and WEINHOUSE, S (1954). *Arch. Biochem* 50, 134.

JEFFRIES, G H (1954). *Quart J exp. Physiol* 39, 77.

JONES, H. B , GOFMAN, J. W., LINDGREN, F. T., LYON, T. P., GRAHAM, D M , STRISOWER, B and NICHOLS, A. V. (1951). *Amer. J. Med.* 11, 358

KAHANE, E and LEVY, J. (1944). *C.R. Ac Sc.,* 219, 431.

KARNOVSKY, M L. and GIDEZ, L I. (1951) *Fed Proc* 10, 205.

KIYASU, J Y., BLOOM, B and CHAIKOFF, I. L. (1952) *J. biol. Chem.* 199, 415.

LAURELL, C. B (1954) *Acta physiol scand.* 30, 289

LE BRETON, E. and PANTALEON, J (1947). *Arch sci. Physiol* 1, 63.

MCARTHUR, C S , LUCAS, C C and BEST, C. H. (1947). *Biochem. J* 41, 612

PERLMAN, I., STILLMAN, N. and CHAIKOFF, I. L (1940). *J. biol. Chem.* 133, 651.

REISER, R , BRYSON, M J , CARR, M. J. and KUIKEN, K. A. (1952). *J. biol. Chem.* 194, 131.

ROBINSON, D S. and FRENCH, J. E (1953) *Quart J exp. Physiol.* 38, 233

ROBINSON, D S , JEFFRIES, G H and FRENCH, J E. (1954) *Quart J exp Physiol* 39, 165.

SCHMIDT, G . HERSHMAN, B and THANNHAUSER, S J (1945). *J biol. Chem.* 161, 523

SCHOENHEIMER, R and RITTENBERG, D. (1936). *J biol Chem.* 114, 381

SHORE, B , NICHOLS, A. V. and FREEMAN, N K (1950). *Proc Soc. exp Biol. Med.* 83, 216

[illegible] (1935) *J. biol Chem* 111, 515

[illegible] 121, 361.

[illegible] *Lab clin Med* 34, 1634

SWANK, R. L. (1951) *Amer. J. Physiol.* 164, 798
[illegible]

WELCH, A. D. and LANDAU, R L. (1942). *J. biol. Chem* 144, 581.
WELD, C. B. (1944). *Canad. med. Ass. J* 51, 578.
ZILVERSMIT, D B, ENTENMAN, C. and CHAIKOFF, I. L. (1948). *J. biol. Chem.* 172, 637.

XX

The Comparative Anatomy of the Larynx with Particular Reference to the Functions of the Organ in Man

SIR VICTOR NEGUS

Introduction

It is necessary, for a full and clear understanding of the functions of the larynx, to approach the subject with a mind unprejudiced by preconceived ideas. This applies particularly to the frequently reiterated description of the larynx as the organ of voice.

If such a view were held, it would materially interfere with a clear interpretation of the many important functions of the organ other than that of phonation, and it would also be detrimental to an easy understanding of the mechanism of phonation.

To attain the necessary degree of mental detachment, it is well to consider the evolutionary factors which have contributed to make the larynx what it is.

Evolution

To seek a new source of food various aquatic animals have, in the course of evolution, ventured on to the land. An example, relevant to the present discussion, is the Climbing Perch (*Anabas scandens*); this fish has a diverticulum of specialized epithelium above the gills, capable of absorbing oxygen, but only if kept moist. This entails return to the water after a brief sojourn on land.

In other, more specialized fish, there is the desirability for breathing air at certain seasons of the year, when the water in which they live dries up in times of drought. These fish would either need to change their habitat, or alternatively they would perish; the problem has been solved in these lung fish, or Dipnoi, by the development of an air breathing apparatus, in the form of a lung. But a pulmonary outgrowth of an efficient pattern must be protected against inundation by water. This protection is afforded by a sphincter at the mouth of the pulmonary outgrowth, the musculature being derived from the pharynx. *This specialized laryngeal girdle is of simple nature*, but is efficient in closing the entrance to the lung when water is in the pharynx (Plate XXI, Figure 1).[1] When the fish is out of water, and needs to breathe air, the sphincter relaxes and allows mouthfuls of air to be forced into the lung by means of a swallowing process.

As evolution proceeds, and air breathing becomes habitual in terrestrial animals, there arises the desirability of active opening of this protective valve, in place of the previous passive relaxation of the muscle band. For this purpose, other fibres, also derived from the floor of the pharynx, assume a dilator function, *to assist in pulling open the slit-like aperture; and to* aid in the efficiency of this action, cartilaginous rods are developed in the margins of the larynx, to which the dilator fibres become attached.

One sees a further stage of evolutionary progress in the division of these lateral cartilages into separate segments, part appearing as the arytenoid cartilage and the remainder fusing with its fellow of the opposite side to form a cartilaginous ring, at first single, but later subdivided into cricoid and thyroid elements. To the arytenoid and to this fused ring, the sphincteric and dilator fibres gain attachment, with added efficiency and with the production of a larynx of the type seen in amphibians, reptiles and birds.

The arytenoid cartilages in reptiles and birds are lengthy, extending along the entire margin of the glottis; they are hinged posteriorly and open as a triangle with the base in the anterior

[1] The plates referred to in this lecture will be found between pages 352–3

position. Such long cartilages do not vibrate readily and cannot produce anything more than a hiss when phonation is attempted.

Having considered the primary reason for the presence of a larynx, it is necessary to continue our investigation as to subsequent changes of structure in relation to other functions.

Olfaction

Although unimportant in man, in most mammals the sense of smell is a vital factor in maintaining survival of the species. In the maintenance of this function the larynx plays an important part, and shows certain modifications which have a bearing, not only on the function of olfaction, but in addition on those of deglutition and respiration.

The primitive larynx described above lies almost flat in the floor of the pharynx; it does not impede the passage of liquids or food into the oesophagus, and, indeed, it partakes in the movements incidental to deglutition, without interfering with that mechanism (*Plate XXI, Figure 1*). Most of the animals possessing this simple type of larynx—amphibians, most reptiles and birds—have no very keen powers of scent; but in most mammals the function of olfaction assumes a high degree of importance, and this enhancement is associated with, and subserved by, certain anatomical characteristics.

In all air-breathing vertebrates the nasal passage communicates with the mouth or pharynx, during respiration air passes through the nose (Plate XXII, Figure 2).

It is essential, if the olfactory sense is to be maintained at a fully efficient level, that air should pass over the olfactory nerve endings in the nose, even when the mouth is open To this end, an epiglottis makes its appearance, and at the same time backward elongation of the soft palate is developed. The result of apposition between the long palate and the projecting epiglottis is the shutting off of the nasal and pharyngeal airway from the food passages, with consequent elimination of mouth breathing when the mouth is open, scent-laden air is not diverted from the olfactory organ, as it would be if no such control of the air current were established

In most keen-scented mammals the epiglottis assumes an intranarial position, lying on the upper surface of the soft palate; but in the dog tribe in particular it lies below the palate, but still with the desired result of obligatory inspiration through the nose.

In man, in contradistinction, vision is relied upon, and the olfactory sense is of little importance and but poorly developed; in him there is no longer apposition of the epiglottis and the soft palate. This results partly from lack of necessity, but mainly because of downward descent of the larynx, consequent on the assumption of the erect posture, with changes in the vertebro-occipital and pituitary angles, and even more through recession of the jaws, which have no longer the prognathous character of most animals and of early man.

The presence of the epiglottis is referred to here, not because of any importance in man in regard to olfaction, but rather because its retention is of considerable significance in the mechanism of deglutition; the separation of the nasal passages from the larynx is of importance in phonation, since sounds are free to pass out through the nose or the mouth

Deglutition

Animals which do not masticate, but bolt their food whole, live mainly on the flesh of insects or other species In them the passage of food is rapid, and can be carried out during temporary cessation of respiration This observation is not universally true, as, for instance, in the case of many snakes, which swallow such an enormous bolus—sometimes even a whole goat or pig—as to require some specialized mechanism. But in carnivorous mammals, it may be taken as a general rule that the larynx is closed during deglutition, without inconvenience. Man, however, although partly carnivorous, is not completely so, and as his evolutionary history shows, his progenitors have passed through a purely herbivorous stage; his larynx shows evidence of this ancestry, and his present habits gain advantage from the evolutionary changes so derived.

Swallowing of liquids. Animals which live on grass, herbage or leaves must swallow very large quantities of food to derive

sufficient nourishment. Deglutition consequently occupies much time; and if respiration had to be interrupted during every movement of deglutition, there would be derangement of the respiratory mechanism. A means has been found, accordingly, to enable liquid or semi-liquid food to pass through the pharynx into the oesophagus, without closure of the larynx and without interruption of respiration. This end is attained by elevation of the margins of the larynx, whereby a lateral food channel is established, along which fluids may travel without inundation of the larynx and trachea. This protective boundary is the aryepiglottic fold, a barrier formed of a mucous membrane covering, with underlying submucous tissues and some contained muscle fibres; the fold stretches from the lateral margin of the epiglottis to the body of the arytenoid cartilage and its superimposed cartilage of Santorini. To assist in maintenance of its protective nature, there is present, in the aryepiglottic fold of many herbivorous animals, and also in man, a supporting prop, the cartilage of Wrisberg. Braced back from behind by tonic contraction of the crico-arytenoideus posticus, the fold provides the medial wall of the lateral food channel. In man it is possible for saliva, or small quantities of other swallowed fluids, to pass into the oesophagus even when the laryngeal aperture is open; in infants this function is even better developed, with considerable advantage, in view of their purely fluid nourishment.

Swallowing of solids. The lateral food channel of man is not wide enough to convey more than very small quantities of fluid at one time, and it would necessitate the drinking of numerous sips if this were to be the habitual method of transmission. Similarly, solid food would have to be carefully masticated and swallowed a very little at a time, if the lateral channel were to be the sole route during deglutition Greater haste is desirable, and therefore means must be found of closing the laryngeal aperture during deglutition; if this is impossible, as it is in certain forms of paralysis such as bulbar palsy, then there will be overflow into the larynx, with immediate cough and possibly with subsequent infection of the pulmonary air tract.

Closure of the larynx is brought about by a somewhat complicated mechanism, executed partly by the intrinsic muscles

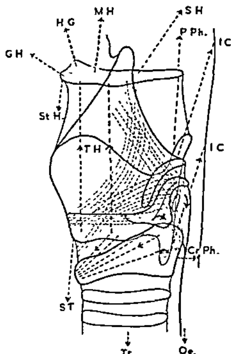


FIG. 3. Direct and indirect action of muscles of the human larynx.

H.G.	= Hyo-glossus	S T	= Sterno-thyroid
G H.	= Genio-hyoid	P Ph	= Palato-laryngeus
S H.	= Stylo-hyoid	Cr Ph	= Crico-pharyngeus
Tr.	= Trachea	I C	= Inferior Constrictor
Oe	= Oesophagus	M H.	= Mylo-hyoid
T H.	= Thyro-hyoid	StH	= Sterno-hyoid

of the larynx itself, and in part by the musculature of the pharynx and hyoid system (Figure 3).

During swallowing of a bolus of any considerable size, whether liquid or solid, there is an initial elevation of the pharynx carried out by its longitudinal or vertical muscles—the stylo-pharyngeus, salpingo-pharyngeus, palato-laryngeus and palato-pharyngeus—together with the elevator and tensor of the palate. In this process of elevation, the larynx takes part, under the influence not only of the muscles referred to, but also of other of the pretracheal group, notably the thyrohyoid (Figure 3).

The larynx rises, not only upwards but also forwards, and is pulled by the muscles attached to the hyoid bone against the base of the tongue, which itself is somewhat recurved during transference of the bolus from the mouth into the pharynx; the thyroid cartilage slides up under cover of the hyoid bone. Being tilted forwards, it follows that the body of the larynx compresses the epiglottis against the base of the tongue; the epiglottis is thus tilted backwards, and appears to turn down as a flap to the laryngeal aperture. It certainly assumes this position, but not with a purposive rôle; animals with no epiglottis, or human beings whose epiglottis has been removed by operation or disease, suffer no disability.

Combined with these movements of the larynx as a whole, there is contraction of the intrinsic sphincteric muscles, and *particularly of the fibres in the aryepiglottic folds, continuous* below with the thyro-arytenoid muscle, and behind with the interarytenoideus (Plate XXIII, Figure 4). The latter, lying below the recurved cartilages of Santorini, *tilts* them forward and so brings the arytenoid cartilages forward towards the cushion of the epiglottis; at the same time, and by the same sphincteric action, the cricoid cartilage is tilted forward on its joints with the inferior cornua of the thyroid cartilage, thus assisting in diminution of the antero-posterior diameter of the larynx.

Closure of the laryngeal aperture, by an inward and forward infolding movement of the flexible aryepiglottic folds, thus takes place in a purse-string manner, to allow this to occur, there is relaxation of the posterior crico-arytenoid muscles, which normally brace back the aryepiglottic folds, and also of the cricothyroid muscles, which pull the thyroid and cricoid cartilages apart.

In considering this physiological process, it is of interest to examine the part played by another muscle arising from the larynx—the cricopharyngeus. The function of this muscle is to keep the mouth of the oesophagus closed, except during deglutition, and thus to prevent air, designed for respiration, from passing into the gullet instead of into the trachea and bronchi.

After elevation of the pharynx, and with it the larynx, as described above, there is a subsequent propulsive peristaltic

wave of contraction, commencing in the superior constrictor muscle and passing down through the middle and inferior components. As the bolus approaches the hypopharynx, a sensory stimulus, initiated in the posterior pharyngeal wall, causes inhibition of the cricopharyngeal sphincter. Relaxation of this muscle not only gives freedom to the upward and forward movement of the larynx, but at the same time allows the mouth of the oesophagus to open in a funnel-like manner; this opening is assisted by drawing upward and forward the anterior wall of the oesophagus, part of which is attached to the recurved tips of the cartilages of Santorini (Figure 3). There is seen, in this mechanism, a very beautiful co-ordination whereby the larynx closes, rises and passes forward so as not to impede the descending bolus and also to prevent the entrance of food into the trachea, while at the same time the oesophagus opens widely to receive the swallowed mass. Lack of co-ordination of these complicated movements sometimes leads, in man, to the formation of a pharyngeal pouch.

It is interesting to recall certain observations as to the position of the cricoid cartilage. It has been mentioned that, during deglutition, relaxation of the cricopharyngeus allows the cricoid to rotate on its joints with the thyroid cartilage, thus facilitating closure of the laryngeal aperture. On the other hand, the cricoid is forced to remain in its more usual position of apposition to the front of the vertebral column during phonation; it is maintained there by the backward pull of the cricopharyngeus. This muscle, arising from the lateral surfaces of the cricoid cartilage, is inserted into a median raphe, which is firmly held back against the prevertebral fascia under the influence of atmospheric pressure, as firmly as though it were attached to the bodies of the vertebrae. Different interpretations are given by others to the relative positions of the cricoid and thyroid cartilages, but on physiological principles, the movements described here appear to correspond with the facts.

Respiration

It is necessary to devote considerable attention to the rôle of the larynx in relation to the physiology of respiration; for the organ,

although only a small element of the tracheobronchial tree, exerts an influence out of proportion to its size.

I have already referred to the essential presence of the larynx as a protector and guardian of the air tract, but its very presence introduces numerous complications, some of which embarrass the very function it is designed to subserve.

Direction of the Air Stream. The primitive larynx has been described as a slit-like aperture lying flat in the floor of the pharynx; its opening is at a right angle to the axis of the trachea, with consequent friction. In the subsequent stages of evolution, with improvement in the rapidity of respiratory exchanges, it is found that a change in inclination occurs. The aperture of the larynx becomes tilted in such a way that the glottis lies almost at right angles to the trachea; in man, with his relatively high aryepiglottic folds, projecting above the floor of the pharynx, the laryngeal aperture inclines somewhat backwards and is, in fact, thus directed so as to fall into line with the current of air arriving from the nasopharynx.

An interesting study may be made of the air streams passing in through the down-turned nostrils of man, then rising through the middle meatus, to descend again past the sloping vault and posterior wall of the nasopharynx, in comparison with the corresponding air currents of animals. As compared with more active and faster running species, the air passage of man is tortuous, with consequent loss of efficiency; but the backward and upward inclination of the laryngeal aperture of man is designed to lessen these deficiencies to a certain extent.

To ensure continuity, and to eliminate points of friction as far as possible, the epiglottis plays its part. Evolved, as already described, for olfactory purposes, the epiglottis of most mammals also plays an important part in respiration, by closing the gap between the soft palate and the larynx (Figure 2). But in man the gap between it and the soft palate leaves an awkward hiatus, where eddies arise and friction reduces the efficiency attained in other species with a continuous airway. But what is lost in olfaction and respiration is gained in phonation, for man, with lack of apposition of palate and larynx, is enabled to emit sounds readily through the mouth, with advantages to

speech. The larynx, in him, has descended in the neck, and this is of still further help in phonation in providing a capacious pharyngeal resonator.

The possibility of mouth breathing in man is made use of in forced respiration, but during buccal respiration alone there is, of course, still greater tortuosity of the inspired air current as it curves round the base of the tongue to reach the laryngeal aperture; there is the compensation of greater efficiency in cooling the body during mouth breathing.

Points of Constriction in the Air Stream. The anterior nares are a place of obstruction; there is a controllable valvular narrowing bounded laterally by the alae nasi. In many animals the muscular control of this valve allows of considerable variation in size of the orifice, but in man the increased capacity attainable is not great. Obstruction at the nares has a considerable relation to the part played by the larynx in allowing passage of air; for at the entrance to the trachea the larynx causes still further obstruction, as it is, in fact, the narrowest part of the pulmonary air tract. It is actually the glottis which causes obstruction, this orifice being of smaller dimensions than either the aperture of the larynx at the level of the aryepiglottic folds, or the subglottic region. The glottis also, of triangular shape when open, offers a cross-sectional area only slightly more than half that of the trachea (Figure 5).

It is necessary now to inquire into the reason for this dual choking at nose and larynx, and particularly into the means by which the opening of the glottis is regulated.

Opening of the Glottis. The dilator muscles have already been mentioned as being of later origin than the sphincteric elements. Arising as fibres derived from the pharyngeal musculature, attached originally to the margins of the glottis, but later with insertion into lateral cartilaginous bars, the dilator or abductor muscles of man work at a mechanical advantage by virtue of their backward migration, with their points of action on the muscular processes and posterior surfaces of the arytenoid cartilages (Plate XXIV, Figure 6). There is thereby a greater effect with less muscular movement than in primitive species. By their action, the posterior muscles rotate the glottic processes of

the arytenoids outwards, and thus separate the vocal folds; but unlike the mechanism in many animals, a further action is executed by outward sliding of the arytenoids on their lax, inclined joints, under pull of laterally placed fibres of the posticus muscle. The glottis is thereby opened in a triangular manner, with its base placed posteriorly; in this, man is unlike most other mammals, in which rotation of the arytenoids gives to the glottis a quadrilateral or diamond shape and in birds a triangular shape with the apex placed posteriorly.

In man the arytenoids are short in relation to the anteroposterior diameter of the glottis, the remainder of the boundaries being made up of the membranous inferior thyro-arytenoid folds, usually known as vocal cords or vocal folds; animals which require rapid and efficient respiratory exchange possess long arytenoids, the optimum length being seven-tenths of the anteroposterior diameter of the larynx (Figure 5).

The shortness of the arytenoids of man is of benefit during swallowing, since the diameter of the laryngeal aperture can, in consequence, be considerably reduced when the sphincteric muscles contract; rotation of the cricoid on the thyroid still further facilitates this shortening, thus permitting a bolus of food to pass readily into the mouth of the oesophagus, opened in a funnel-like manner to receive it.

The decrease in length of the arytenoid cartilages permits the membranous folds to be so much the longer, with advantages in phonation and fixation of the thorax.

It must be observed that choking of the airway at the larynx is not necessarily an accompaniment of shortening of the arytenoids; if a more capacious glottis were required, it could be provided by increase in size of the larynx as a whole in the anteroposterior direction, as seen in many animals It is of interest to note that this relative increase is present in the human foetus and in infants, in whom the posterior plate of the cricoid cartilage has a backward inclination, thus giving to the larynx somewhat of a funnel shape and bringing its cross-sectional area nearer to that of the trachea. In the male at puberty a similar increase is obtained, by growth changes affecting the anteroposterior diameter (Plate XXV, Figure 7).

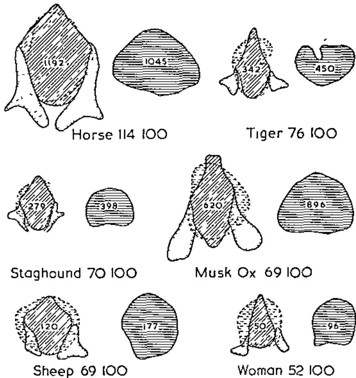


FIG 5 Transverse sections of glottis and trachea The outline of the [illegible]

The enlargement of the larynx is very obvious in many animals such as members of the dog tribe.

Conclusions. Man relies on his intelligence for survival, rather than on his physical capabilities. He is neither so strong as the gorilla, orang or chimpanzee, nor so fleet as the cheetah, the gazelle or the horse. A man can run at about 20 miles an hour, a gazelle at 60 and a cheetah at 70 for short distances. And yet man has an ascendancy over these animals.

For reasons to be given later, obstruction at the nose and

larynx is put to advantage for certain purposes of respiratory exchange, but always with an accompanying disadvantage of limitation of exertion. Obstruction at the nose can, to a certain extent, be counteracted by breathing through both nose and mouth; and this route is adopted by a runner, with advantage, not only with increased respiratory exchange, but also with increased cooling of air, since the warming effect of the nose is correspondingly reduced.

Tortuosity of the air passages can be diminished slightly by extension of the head on the spinal column, as may be noted in a sprinter; but under no circumstances can the air tract be straightened out, as it is in most fast-running animals.

It is to be observed, therefore, that man, being versatile and with high development of certain faculties, suffers the disadvantages of a somewhat inefficient respiratory apparatus without reduction of his general superiority; for the loss in one direction is compensated by a gain in others.

Respiratory Movements at the Glottis

The choking at the glottis is only of disadvantage at times of severe exertion; at times of gentle exercise, or during resting periods, the aperture offers no undue obstruction. On the contrary, there are to be seen at the glottis movements of dilatation and constriction during respiration, and their purpose must be considered.

It has already been observed that the anterior nares delay the incoming air, measurements of air pressures in the nasopharynx showing a fall below that of the atmosphere during inspiration, and a rise on expiration. But there is a still further rise and fall in the trachea, by virtue of obstruction at the glottis. Observations show a widening of the glottis at the immediate commencement of respiration, more noticeable during some degree of exertion than at times of complete repose; and correspondingly, a narrowing during expiration, again sudden in its execution and slightly in advance of the movements of the chest wall. The reason for these rhythmic movements appears to depend on the necessities of respiratory exchanges in the lungs. Oxygen must be taken up by the blood

from the air in the alveoli, and carbon dioxide must be given off; and these exchanges must be carried out rapidly, during the passage of blood through the pulmonary capillaries.

Pump Action. The larynx exercises two functions. The first is directed to assist the circulation by a pump action. Partial obstruction of the glottis during inspiration assists in lowering intrathoracic pressure, and this in turn reduces pressure on the great veins and right auricle. A suction action is thereby brought into play, with attraction of blood from the systemic circulation into the right side of the heart. The same reduction of pressure leads to dilatation of the pulmonary capillaries, with reduction of obstruction to the flow of blood through the lungs; in consequence more blood reaches the left auricle, and according to the law of the heart, an increased volume leaves the left ventricle, with a rise of aortic pressure (Figure 8).

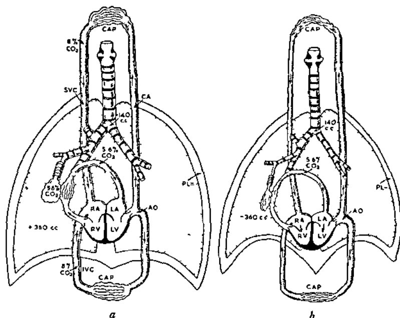


FIG. 8 Diagram to illustrate (*a*) the dilatation of the pulmonary capillaries during inspiration, and (*b*) their collapse during expiration The condition of the glottis and of the bronchiolar musculature is seen in the two phases.

Regulation of Respiratory Exchanges. This degree of acceleration of the blood flow during inspiration must be regulated in accordance with the amount of air reaching the alveoli; for it is necessary that there should be sufficient freshly replaced air in the lungs for the respiratory exchanges. If air entered rapidly and left in too great volume, there would be insufficient time for giving up oxygen and taking up carbon dioxide, with waste of energy; on the other hand, if the tidal air were excessively reduced, and a diminished volume reached the alveoli, the large quantity of blood passing through the lungs under the influence of the reduced intrathoracic pressure would pass into the systemic circulation insufficiently charged with oxygen and overcharged with carbon dioxide.

It is thus possible for the size of the glottis to correlate the amount of inspired air and the quantity of blood in the lungs to the best advantage, and this seems to explain the movement of glottic widening seen at the beginning of inspiration. When expiration commences, the glottis narrows, and this again appears to have a purposive meaning. For during expiration, when air overcharged with carbon dioxide and deficient in oxygen is escaping from the lungs, the efficiency of respiratory exchange is much diminished; it leads to conservation of energy, therefore, if the pumping of blood through the collapsing lungs is reduced during expiration. This is attained by increase of pressure in the thorax, and this again depends to a certain extent on obstruction at the glottis If, therefore, there is partial closure of the vocal folds at the beginning of expiration, with slowing down of the expiratory air stream, there will be a corresponding slowing of the intrapulmonary blood flow, the [illegible]

blood when under water.

It is of interest to note that after tracheostomy there is an increase in the respiratory rate, and also a diminution in the powers of muscular exertion in certain actions; but other factors, later to be considered, also have an influence in this direction.

Elimination of Dead Space Air. It is necessary to refer to the possibility of participation of the larynx in this function, even though the conclusion will be negative. An important factor in respiration is the help given by expiratory contraction of the bronchiolar, bronchial and tracheal musculature in squeezing air out of the dead space of the tracheobronchial tree, preparatory to the inspiration of a fresh and uncontaminated volume of tidal air; this action is one of importance in many animals, and in it most parts of the airway participate, including the nose, where contraction of the alae nasi may be observed at the end of expiration. In this process of clearance it might be thought that the larynx played a part, and that the expiratory adduction of the vocal folds was designed to expel used-up air. But such is not the case; for observations show that opening and closing of the glottis occur at the extreme commencement, or even just before, the descent and elevation of the diaphragm. Far from removing expired air, the larynx is thus seen to have a contrary effect of delaying the air current; the purposes of this obstruction have already been studied.

Function of the Saccules. In certain animals, particularly some amphibians, reptiles, birds and mammals, air sacs are present on the pulmonary air tract, the purpose appearing to be directed to rebreathing of air, this mechanism is seen at its

In some of the higher apes the laryngeal saccule is highly developed, and is continuous with large air sacs, which reach, in certain species, even into the axilla. In man the saccule is a small upward extension of the laryngeal ventricle and is of insignificant size.

It might be thought that the saccule in man had a purposive function, but such is not the case. Even when the saccule is abnormally enlarged into a laryngocele, projecting well above the thyroid ala, as is sometimes seen in the human species, there is no evidence to denote any connection with an increased efficiency of the pulmonary mechanism.

Regulation of Intrathoracic Air Pressure

In addition to its important function of helping to control the air-stream as it enters and leaves the lungs, there is another aspect of the valvular glottis to be considered; this concerns complete interruption, either of inspiration or expiration, as a means of assisting fixation of the thorax.

Prevention of Entrance of Air. The simple sphincteric larynx of the lung fish is not well designed to resist high degrees of alteration in air pressure, but in certain amphibians and reptiles, such a valvular type is present. In herbivorous types, elevation of the margins of the larynx, with the creation of a lateral food channel, leads to the formation of an organ which is of non-valvular nature in those species with a purely terrestrial habitat. But if the larynx of herbivorous animals with arboreal habits be examined, it will be observed that valvular folds are present in the margins of the glottis; these folds are upturned and in many instances are of an efficient valvular type, somewhat similar to the aortic valves of the mammalian heart. It is obvious that these valvular folds, if brought into mutual apposition, will efficiently prevent air from entering the trachea. An examination of the larynx of all mammals shows that the possessors of this type of larynx have similar habits of feeding on berries, nuts or leaves, and of making independent prehensive use of the fore-limbs for grasping or climbing (Figure 9).

Man shows a similar structure, although to a somewhat less efficient degree; in him secondary inferior thyro-arytenoid folds have been evolved as fixation valves, to be used for a purpose similar to that of arboreal animals, as will shortly be explained.

When an animal hangs on to the branch of a tree, or pulls itself up by the fore-limbs, or when it hugs—as in the case of a bear—or when a man lifts a heavy weight, the pectoral muscles play an important part. These muscles arise from the ribs, and are inserted into the humerus; as they contract they can either elevate the ribs, as in forced respiration, or alternatively they can depress or adduct the fore-limb. When the latter action is required, means must be found of immobilizing the ribs, if the muscle is to function efficiently. The ribs can be held down by

muscular action, executed mainly by the muscles of the abdominal wall, but assistance can also be given through the intermediary of the larynx. If the glottis be closed at the beginning of the fore-limb effort so as to prevent air from entering the trachea, then any tendency to expansion of the thorax will be

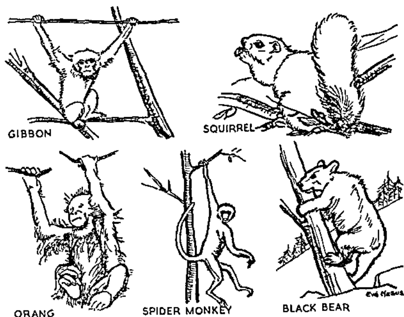


Fig. 9 Use of forelimbs for hanging and climbing in Orang Utan (*Simia satyrus*), for swinging from branch to branch in Gibbon (*Hylobates*) and Spider Monkey (*Ateles*), for holding food and climbing in the Squirrel (*Sciurus*), and for climbing and hugging in the Bear (*Ursus*).

counteracted, and resistance will be offered to elevation of the ribs; it is, of course, necessary that during this action the diaphragm should contract, as in an ordinary inspiratory effort.

This, then, appears to be the primary function of the inferior thyro-arytenoid fold, such as that of lemurs, monkeys, apes and man, the fold is used also for phonation, and is therefore generally referred to as the vocal cord, even though its use for this purpose is a later and superadded function.

It is of interest to note that fixation of the thorax in this position of inspiration, with temporary reduction of intrathoracic pressure, is of physiological advantage, in that it assists in the pump action of the heart and facilitates the intrapulmonary circulation.

Not only does the evidence of comparative anatomy support the explanation advanced, but there is also corroboration to be obtained by direct observation. If a man be asked to compress *a spring balance with his arms held forward, and if the larynx* be observed at the same moment, it will be seen that the vocal folds come together with a snap, the ventricular bands remaining apart and not obscuring the view of the lower folds. Radiographic examination shows inspiratory descent of the diaphragm during this action, with but slight elevation of the ribs. Even during severe exertion of this type, the individual does not become cyanosed, owing to the accelerating effect on the circulation referred to above.

Function of Laryngeal Ventricle. The ventricle comes into existence as a result of the formation of this secondary fixation valve; it is left as a recess lateral to the inferior thyro-arytenoid fold, lined by an epithelial membrane, beneath which is placed part of the thyro-arytenoid muscle. Numerous glands secreting mucus are situated in the epithelium and submucosa, with the function of lubricating the adjacent fold and maintaining its free edge in a supple and efficient condition.

Reference has already been made to the saccule, which in man appears as an upward extension of the laryngeal ventricle. It is not, however, of the same purposive derivation as the latter; its function has been discussed above.

Prevention of Escape of Air. Under certain conditions the larynx assists in maintaining the intrathoracic pressure at a level in excess of the normal. This occurs during straining movements, at times when pressure has to be exerted on the abdominal viscera, as in defaecation. Compression by the anterior abdominal wall would naturally tend to elevate the diaphragm, and resistance to such movements can be assisted by the imprisonment of air in the lungs. To this end the superior thyro-arytenoid folds, usually known as ventricular bands, are brought

together by contraction of the sphincteric group of intrinsic laryngeal muscles; the action entails contraction of certain upper fibres of the thyro-arytenoid group, lying to the outer side of the ventricle, together with contraction of the inter-arytenoideus. The folds, with their down-turned margins, are able to resist a very considerable pressure of air if the chest be filled and the thoracic walls compressed.

The mechanism is physiologically unsound, in so far as it leads to compression of the great veins, the right auricle and the pulmonary capillaries, with consequent slowing of the circulation and damming up of blood in the right side of the heart (Figure 8). An indirect effect is produced on the aortic circulation, because the left auricle, receiving a reduced volume of blood, transmits less to the left ventricle; according to the law of the heart, the latter pumps less blood into the aorta and the systemic arterial pressure falls.

During severe straining efforts of this type, the individual may show a degree of cyanosis, unlike the pink or sometimes pale colour present when the mechanism of reduced pressure is invoked; the latter, as already pointed out, is thus physiologically superior in efficiency.

Protection of the Tracheobronchial Tree Against Damage by Foreign Bodies

The larynx plays an important part in this respect, and some detailed consideration is desirable.

Protective Closure. In the first place, the primitive function of the larynx must be recalled The organ was evolved as a valve to prevent inundation of the trachea and lungs by water or by food. In lower forms, such as amphibians, fish, reptiles and birds, closure is executed at the glottis, which is bounded by a muscular sphincter, with or without the assistance of lateral cartilages; the glottis thus forms the laryngeal aperture (Plate XXI, Figure 1). In many mammals a similar arrangement is seen, as in members of the cat tribe. Here there is a simple type of larynx, with glottic boundaries made up partly of membranous folds and partly of arytenoid cartilages, bounded laterally and actuated by a group of muscles.

In man the glottis is described as the aperture between the secondary thyro-arytenoid folds, consisting of ventricular bands and vocal folds. It is not here that protection against inundation is afforded, the function having been assumed by those folds superjacent to the glottis—the aryepiglottic folds. The method of their closure has already been described; it has also been pointed out that these upstanding folds form a barrier against fluids descending along the lateral food channel, even when the larynx is not closed. But although the aryepiglottic folds are thus efficient in excluding water and food, yet they do not keep out light objects carried by the inspiratory air current; these objects may be particles of dust, bacteria, fragments of food, and occasionally other and larger foreign bodies, which can lead to obstruction or infection of the lungs.

The glottis is the narrowest part of the pulmonary air tract. Any object, therefore, which passes through the larynx will be of insufficient size to obstruct the trachea; it may block up a bronchus, but will not directly fill the trachea.

It might be said, therefore, that although the larynx may sometimes fail in its function of effectively protecting the pulmonary air tract, yet it has a selective action in excluding objects which may produce immediate asphyxia; it is, of course, possible for such large objects to become impacted in the larynx itself and to produce death.

But as regards prevention of infection, it is obvious that the protective closure of the laryngeal aperture is of little avail.

To consider the alternative available methods of protection if the larynx has failed in its guardianship, it is necessary to turn attention first to the protective action of cough.

Cough. The mechanism of cough resembles that described above for the raising of intrathoracic pressure, except that the positive pressure is sustained for a very short period only. Preparatory to cough an inspiration is first taken, and the glottis is then closed, with apposition of the ventricular bands in addition to the vocal folds. An expiratory effort is next made, the intratracheal pressure being raised very considerably. As soon as this rise has been attained, the glottis is opened by relaxation of the sphincteric and contraction of the dilator muscles; the

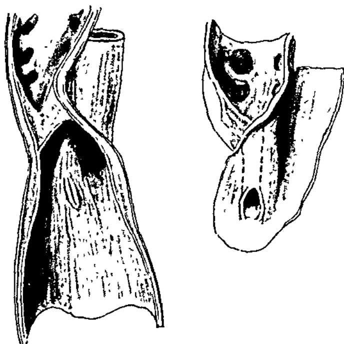


FIG 1 Larynx of Australian Lung Fish (*Ceratodus*) Closed on the left and open on the right It lies flat in the floor of the pharynx, The oesophagus is seen passing up to the right and the saccular lung to the left in each drawing (This and subsequent illustrations appeared in the author's *The Comparative Anatomy and Physiology of the Larynx*, 1949, London, Heinemann)

In man the glottis is described as the aperture between the secondary thyro-arytenoid folds, consisting of ventricular bands and vocal folds. It is not here that protection against inundation is afforded, the function having been assumed by those folds superjacent to the glottis—the aryepiglottic folds. The method of their closure has already been described; it has also been pointed out that these upstanding folds form a barrier against fluids descending along the lateral food channel, even when the larynx is not closed. But although the aryepiglottic folds are thus efficient in excluding water and food, yet they do not keep out light objects carried by the inspiratory air current; these objects may be particles of dust, bacteria, fragments of food, and occasionally other and larger foreign bodies, which can lead to obstruction or infection of the lungs.

The glottis is the narrowest part of the pulmonary air tract. Any object, therefore, which passes through the larynx will be of insufficient size to obstruct the trachea; it may block up a bronchus, but will not directly fill the trachea.

It might be said, therefore, that although the larynx may sometimes fail in its function of effectively protecting the pulmonary air tract, yet it has a selective action in excluding objects which may produce immediate asphyxia; it is, of course, possible for such large objects to become impacted in the larynx itself and to produce death.

But as regards prevention of infection, it is obvious that the protective closure of the laryngeal aperture is of little avail.

To consider the alternative available methods of protection if the larynx has failed in its guardianship, it is necessary to turn attention first to the protective action of cough.

Cough. The mechanism of cough resembles that described above for the raising of intrathoracic pressure, except that the positive pressure is sustained for a very short period only. Preparatory to cough an inspiration is first taken, and the glottis is then closed, with apposition of the ventricular bands in addition to the vocal folds. An expiratory effort is next made, the intratracheal pressure being raised very considerably. As soon as this rise has been attained, the glottis is opened by relaxation of the sphincteric and contraction of the dilator muscles; the

PLATE XXI

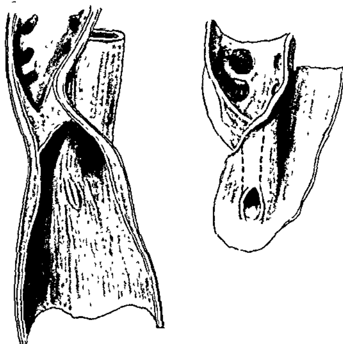
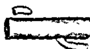


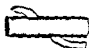
FIG 1 Larynx of Australian Lung Fish (*Ceratodus*). Closed on the left and open on the right It lies flat in the floor of the pharynx, The oesophagus is seen passing up to the right and the saccular lung to the left in each drawing (This and subsequent illustrations appeared in the author's *The Comparative Anatomy and Physiology of the Larynx*, 1949 London, Heinemann)

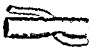
PLATE XXII



















[illegible]

nd the larynx and the role of the epiglottis in closing the intervening gap
Drawing by D Tompsett)

Plate XXIII

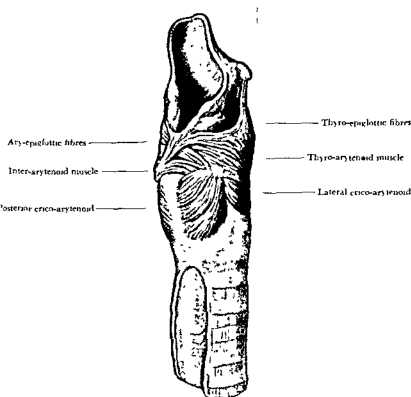
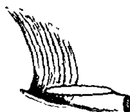
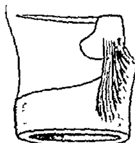


Fig 4 Dissection of larynx of a man (Museum of Royal College of Surgeons, England) The dilator muscle and the components of the sphincteric group are illustrated.

PLATE XXIV







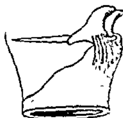


FIG 6 Migration of dilator muscles In the African Lung Fish (*Protopterus*) circular muscle fibres of the pharynx run below and partly into the margins of the glottis at its anterior end The Alligator has dilator fibres arising from the crico-thyroid ring and inserted partly into the tip of the long arytenoid cartilage and partly into the margin of the glottis anteriorly The Lioness (*Felis leo*) has a fan-shaped dilator muscle capable of rotating the short arytenoid cartilage and of pulling it outwards In Man, the arrangement is similar to that of the Lioness.

PLATE XXV

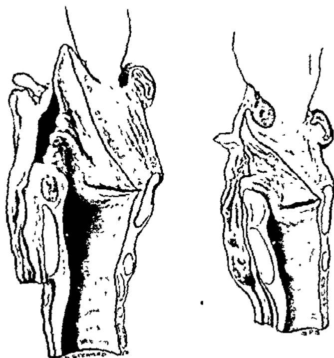


FIG 7 Growth changes in the adult human larynx (Natural size) *Left* Male, nineteen years *Right* Female, fifteen years The marked elongation of the antero-posterior diameter of the glottis, occurring at puberty, is illustrated by the larynx of the young man, the glottis thus becomes less of a choked point on the airway The female larynx retains the characteristics of the juvenile type, with short arytenoid cartilages, relatively long thyro-arytenoid folds and a relatively short antero-posterior diameter

imprisoned air escapes with an explosion, carrying out with it any collection of mucus or a small foreign body, such as a fragment of food which has inadvertently been aspirated into the trachea.

Although of physiological importance, yet cough is not the habitual method of ridding the tracheobronchial tree of excess of mucus or bacteria; it is almost a pathological process and may not be employed from the beginning to the end of the year, unless some abnormal conditions arise, such as inflammation of the tracheal or bronchial mucosa. The more important mechanism of ciliary action will be referred to below.

Cough as it affects the larynx has points of considerable interest. It is a reflex process, depending on stimulation of the sensory nerve supply, with referred stimulation of the expiratory centre. The nerves involved are the glossopharyngeal in the region of the epiglottis and aryepiglottic folds, the internal laryngeal branch of the superior laryngeal nerve in the area of the larynx above the vocal folds, and the recurrent laryngeal nerve in the sub-glottic region. Cough is of brief duration, and therefore not to be relied upon. If a small foreign body becomes impacted in the larynx, cough will result, but only for a brief period, for after possibly no more than a minute the effort ceases

valvular manner is lost, and effective cough becomes impossible; such efforts as are then executed resemble the cough of a cow, in which there are no valvular folds similar to the ventricular bands of man The individual affected is then said to have a bovine cough.

Similarly, if tracheostomy has been performed, the ability to raise the intratracheal pressure by virtue of closure of the ventricular bands is lost, and cough here again is ineffective.

Cough is able to remove a bolus of mucus with entangled bacteria, but it cannot materially affect the layer of mucus habitually covering the mucosa of the trachea, bronchi and larynx. Every breath we draw carries with it some floating organisms, particularly in droplet form, more so in the proximity

of an infected individual and particularly at times of epidemics of upper respiratory infections. The nose removes some bacteria, but not all, and therefore it must be assumed that many pass the larynx. Protective closure against their entrance is impracticable, and cough removes no more than those entangled in a blob of mucus; a further defensive mechanism is ciliary action.

Ciliary Action. Although this lecture is concerned with the larynx, and therefore does not embrace the whole subject of ciliary action, yet there are certain points which must be referred to.

The ciliary streams have been extensively studied in the bronchi and trachea, and their importance has been recognized in effecting the removal of bacteria which have escaped the defences of the nose and have passed through the larynx during inspiration. The blanket of mucus covering all the mucosa is essential to health, and if it is in effective functional activity, there will be little possibility of invasion of the underlying mucosa by pathogenic organisms.

As the ciliary streams meet in the trachea after leaving the bronchi, they adopt a somewhat spiral course, but in the trachea itself they are distributed over the whole mucosa. If cough arises, however, there is considerable contraction of the elastic posterior wall of the trachea under muscular action, the lateral and anterior walls being more rigid, by virtue of their cartilages. It is to be assumed, therefore, that the covering mucus will tend to be squeezed away from the posterior wall. But when the streams reach the subglottic region, they must alter course and must converge in the posterior region. The reason for this change of direction is the fact that the vocal folds are covered with squamous epithelium, while the subglottic and the inter-arytenoid regions have a ciliated epithelium.

It is only through the inter-arytenoid space that the stream of mucus can pass to reach the pharynx, to be removed thence by swallowing movements.

It is easy to verify this theoretical assumption by direct observation. The visible secretions of mild bronchitis, or those associated with pulmonary tuberculosis, are concentrated into

this posterior gap and may be seen cascading over the lip of the laryngeal aperture. In tuberculous laryngitis the lesions are usually posterior in distribution, and the direct deposit of tubercle bacilli, which are present in the sputum of the majority of patients who develop laryngeal lesions, accounts for this phenomenon.

Although mucus is not conveyed on to the surfaces of the vocal folds from below, yet it reaches them from above, there being many mucous glands in the mucosa of the laryngeal ventricles; the squeezing action of the sphincteric muscles of the larynx carries mucus on to the folds, where it has an important function in lubrication, essential for efficient phonation.

Phonation

It will be noticed that this subject has been left to the last, as a function of no comparable vital importance to those connected with respiration and deglutition.

Almost any larynx serves for the production of sound if air be blown through the glottis. A simple illustration is an artificial larynx constructed of two small strips of muscle from the thigh of a frog, pinned side by side over an aperture connected with a pressure pump. If air be blown through, the strips vibrate and a sound is produced, rising in pitch if the elasticity of the muscle is raised by faradic stimulation; the sound is louder if a resonating chamber is applied to the preparation.

This experiment illustrates the mechanism of phonation at the larynx; the lungs are the source of air pressure, the inferior thyro-arytenoid folds act as the vibrating reed, and the pharynx, mouth and nasal passages are the resonators.

The glottis of a reptile or bird is not well adapted for sound production, because of the long and rigid arytenoid cartilages; the glottis of a cat, bounded by undivided thyro-arytenoid folds, is admirably suited for phonation and is made good use of.

The inferior divisions of the divided thyro-arytenoid fold of man, with short arytenoid cartilages and long membranous folds, bounded by thyro-arytenoid muscles of variable elasticity, give to man an organ well suited to the production of sounds of wide pitch and satisfactory quality (Figures 5, 10).

The thyro-arytenoid muscle is considered as consisting of an internal and an external division; attachment to the body of the arytenoid cartilage enables the glottic margin to be shortened in the absence of opposition by the posterior crico-arytenoid muscle, and also allows of inward rotation of the glottic processes of the arytenoid cartilages. Apposition of these cartilages is assisted by a rotating action of the lateral crico-arytenoid, and by a movement of approximation by the interarytenoid muscle (Figure 3, Plate XXIII, Figure 4).

The other intrinsic muscles of the larynx act in unison with the thyro-arytenoids, to regulate the length of the glottis and the degree of elasticity, in relation to the pressure of air escaping from the trachea. The position of the larynx is regulated by the various muscles attached to it (Figure 3).

The glottic margins alternately separate when blown apart and recoil when the intratracheal pressure is thereby reduced; rhythmical vibration produces sounds of fixed pitch and these sounds are then amplified and modified by the resonators.

Certain overtones may be accentuated to give various patterns to the laryngeal sounds, by virtue of alteration in shape and size of the superimposed resonators, namely the pharynx, mouth and nasal cavities; in this way the different vowel sounds can be distinguished by the ear.

Consonants are produced by the modification of laryngeal sounds, amplified in the resonators and given the requisite character by alteration at the various stops provided by the tongue, the lips, the teeth and the soft palate.

From the original involuntary sounds, later adapted to purposive cries and still further elaborated into vowels and consonants, language is built up A large buccal cavity, bounded by cheeks evolved for mastication and closed by lips provided for suckling, confer considerable advantages.

Man's intelligence has allowed him to take advantage of the laryngeal sound-producing organ so as to elaborate a code of communication; this ability to convey messages and ideas to others has in its turn increased his intelligence and has placed him in a commanding position in the animal kingdom.

Reduction of the length of the arytenoid cartilages with

elongation of the membranous folds, separation of the epiglottis from the soft palate as the result of descent of the larynx [illegible] man a vocal apparatus of great efficiency. Regression of certain characteristics, such as speed in running, keenness of sense of smell and arboreal activity, have been compensated by general versatility.

It is interesting to note that if the larynx is removed surgically, usually because of malignant infiltration, it is possible for the subject to evolve a new mechanism of phonation. The crico-pharyngeal sphincter, which guards the mouth of the oesophagus, is utilized for sound production; it is made to vibrate under the influence of air taken into and then expelled from the oesophagus, with the production of mixed sounds. These sounds, corresponding roughly to those normally produced by the larynx, are amplified and modified by the resonators referred to above, in such a way that audible and intelligible speech can be carried on.

This is but one example of the observation that a very simple sound-producing organ can be put to excellent use, provided the individual has the intelligence to avail himself of the apparatus.

Conclusion

The evolution of the larynx has been outlined and its various modifications have been described in relation to the many and varied functions related to its use. Man has taken advantage of an organ modified by many factors, and is thus enabled to elaborate a wide range of vocal signals, built up into a vocabulary, his intelligence in availing himself of this possession has been a powerful factor in his intellectual advancement

XXI

Histochemistry and its Application to the Basic Sciences

A. G. EVERSON PEARSE

Introduction

In both the old and new worlds the unqualified term histochemistry has come to mean tissue section, or microscopic, histochemistry. This means the localization in relatively intact tissues and cells of chemically or physically identifiable substances and reactive groups, as opposed to their localization in extracts or homogenates and to their chemical estimation in disrupted tissues. At least one authority on the subject (Lison, 1953) maintains that histochemistry must mean this if it is to be regarded as a separate science; otherwise, he says, it becomes merely biochemistry.

All methods of evaluating the structure and function of cells come properly under the broad heading of *Histochemistry*, yet many obviously belong to the sciences employing them. Up to the present, therefore, I have tried to avoid thinking and speaking of histochemistry as a separate science. There is no doubt, however, that 'Microscopic Histochemistry', the term used by Gomori (1952) as the title of his book on the subject, tends more and more to become a separate science of its own This is partly due to the complexity of its techniques which are nowadays by no means always derived directly from chemistry and biochemistry.

In this lecture I propose to deal with that collection of methods essentially applicable to the type of material, be it tissues or cells, with which the conventional studies of many of the basic sciences are carried out. One is thinking particularly

of *Histology*, *Cytology*, *Haematology*, *Embryology*, *Zoology*, *Botany* and *Pathology*. Other sciences, *Bacteriology* and *Mycology*, for instance, have on the whole been less concerned with histochemical methods, as have *Physiology* and *Pharmacology*. Still others, such as *Enzymology* and *Biochemistry* seldom or never employ histochemical methods. With these the exchange is entirely in the opposite direction. The greatest advantage of microscopic histochemistry, and the main reason for its employment, is the fact that it is able to distinguish and delineate the structure and function of one cell from that of its immediate neighbour. Of secondary importance is the comparative ease with which a large number of methods may be applied to a large number of different types of tissue. The greatest disadvantages of microscopic histochemistry, at the present time, are that it follows, but never quite catches up with, the advances of contemporary biochemistry and that quantitation of its essentially qualitative methods with any degree of accuracy is possible in only a few instances.

Historically histochemistry is as old as the science of histology but, although isolated reports of the chemical investigation of structure appeared at the beginning of the nineteenth century, the first clear appreciations of microscopic chemistry came from the French pharmacist F. V. Raspail, originator of the liqueur and the pharmacy which bear his name and recipient of the honour of having a boulevard in Paris called after him. Writing in the early 1830's he expressed the aims and objects of histochemistry in terms which are applicable today.

At first, histochemistry was largely a botanical science and in its application to animal tissues it was essentially destructive. As such it continued, largely submerged by the mass of new colouring techniques which arose between 1862 and 1900, following the discovery and application of the aniline dyes In this period Paul Ehrlich, Frederick Miescher and, a little later, Gustav Mann, kept the young science of histochemistry alive. In 1936 occurred the renaissance of tissue histochemistry with the publication by Lison of his great work *Histochimie Animale*. Lison, without doubt, must be regarded as author of the new science of histochemistry without tissue destruction.

The Techniques of Modern Histochemistry

In the years following 1936 histochemical methods were used with increasing frequency by workers in various fields and, since 1949, interest in the applications of histochemistry has grown enormously. At the same time a large number of new techniques have been introduced. In the tables which follow the various histochemical methods for broad groups of substances are set forth, and these have been divided, in most cases, into those practised before 1945 and those developed after 1945. In the case of the enzymes, however, divisions have been made at *1900 and 1939 in order to emphasize the extraordinary* development of methods in this category following the impetus

Table 1. Protein Methods
('End Group' and others)

Up to 1945	
Xanthoproteic	OH-phenyl
Millon	OH-phenyl
Diazonium	Arom: AA[3]
Voisenet-Furth	Tryptophan
Ninhydrin	NH_2
Alloxan	HN_2
Sakaguchi	Arginine
Lead acetate	SS
Nitroprusside	SH
Ferricyanide	SH
Since 1945	
Tetrazonium	Aromatic AA[3]
DNFB (di-nitrofluorobenzene)	NH_2
8-Hydroxyquinoline	Arginine
Azomethine	NH_2
Ninhydrin-Schiff	NH_2
Alloxan-Schiff	NH_2
β-naphthaldehyde	NH_2
Phenacylbromide	SH
RSR (Red sulphydryl reagent)	SH
Tetrazolium	*SS and SH*
D.D.D (di-hydroxy di-naphthyl disulphide)	SS and SH
β-naphthyl maleimide	SH
Fluorescent A–B methods	

given when Gomori and Takamatsu, in 1939, independently introduced their essentially similar methods for alkaline phosphatases.

In the case of the protein methods modern advances have

TABLE 2. Carbohydrates

Up to 1945	
Metachromasia	Acid mucopolysaccharides
Bauer-Feulgen	Glycogen
Iodine	Glycogen
Best's Carmine	Glycogen
Silver methods	Glycogen
Chlor-zinc-iodine	Chitin and Cellulose
Iodine-sulphuric acid	Cellulose
Mucicarmine	Mucins
Mucihaematein	Mucins
Silver nitrate	Ascorbic A.
Ptyalin extractions	
After 1945	
Periodic acid-Schiff	1 : 2-glycols
Alkaline tetrazolium	Chitin and alkali-fast sugars
Alcian Blue	Sulphated mucins
Methylene blue extinction	Mucopolysaccharides
Dialyzed iron	Mucopolysaccharides
Evans blue	Mucopolysaccharides
Sulphation methods	
Hyaluronidase	
Diastase	Extractions
Pectinase	

been largely academic but some of the new techniques are of sufficient reliability for routine application to protein studies. In particular the Ninhydrin-Schiff and Alloxan-Schiff methods (Yasuma and Itchikawa, 1953) represent a great advance on the older methods for NH_2 groups. The whole group of new methods for sulphydryl and disulphide groups has led to *renewed interest in the histochemistry of the keratins and of the* neurosecretory material of the hypothalamus and posterior lobe of the pituitary gland, since oxytocin and vasopressin have been shown to be cystine-containing octapeptides (Tuppy, 1953; du Vigneaud, Ressler and Trippet, 1953). The fluorescent

antibody methods, introduced by Coons and his collaborators (Coons, Creech, Jones and Berliner, 1942), have been used successfully by several groups of workers (e.g. Marshall, 1951;

TABLE 3. Enzymes

Before 1900		*Before 1939*	
Peroxidases	Guaiac	Peroxidases	Benzidine and zinc-leuco
Indophenol (Cytochrome) oxidase		Phenoloxidases	α-naphthol
		DOPA-oxidase	Bloch-Laidlaw
		Myrosulphatase	Peche

Hill and Cruikshank, 1953; White, 1954). They depend on the production of a specific antibody and on the coupling, *in vitro*, of the ε-NH_2 groups of its constituent lysine molecules with fluorescein isocyanate. The resulting fluorescent antibody complex attaches itself to the antigen in tissue sections (freeze-dried or fresh-frozen), when applied to them, and the sites of attachment are revealed by fluorescence microscopy. At present the application of Coon's particularly elegant technique is beset with numerous problems of a purely technical nature.

With the exception of the fluorescent antibody methods the techniques of protein histochemistry have limited application or, rather, the information derived from their use is of limited value.

Although the number of new carbohydrate techniques has been considerably fewer than those in the protein field one in particular (the periodic acid-Schiff method of McManus, 1946) has proved to be of exceptional value. Some of its many applications I propose to illustrate and discuss in the final section. Second in importance to the PAS technique is the use of Alcian blue (Steedman, 1950; Pearse, 1953a) for demonstrating sulphated mucins. The alkaline tetrazolium method (Pearse, 1953b) is also capable of giving useful information about the various mucins, especially when used in conjunction with the above methods.

From Table 3 it is evident that up to the year 1939 the only

significant enzyme methods were those demonstrating oxidases. A single hydrolytic enzyme (myrosulphatase) appears in the list to emphasize the complete failure of histochemistry in this field.

TABLE 4 Enzymes

After 1939		
Enzyme	No. of Methods	Types
Alkaline Phosphatases	3	Ca-CoS, Azo dye, Indoxyl
Acid Phosphatases	3	PbS, Azo dye, Indoxyl
5-Nucleotidase	1	Ca-CoS
Aldolase	1	$MgNH_4$-CoS
ATP'ase	1	Ca-CoS
Glucose-6-phosphatase	1	PbS
Phosphorylase	1	Glycogen-PAS
Phosphamidase	1	PbS
Lipase	1	Tween
Esterases (non-specific)	4	Azo dye, Indoxyl, Tween, Myristoyl
A.S. Esterase	1	Azo dye
Chloroacetylesterase	1	Azo dye
Cholinesterases	7	Azo dye, Indoxyl, Myristoyl choline, carbonaphthoxycholine, Acetylthiocholine, Butyrylthiocholine, Thioacetate
Phenolsulphatase	3	Azo dye, PbS, Benz: naphthoquinone
β-glucoronidase	3	Ferric-hydroxyquinoline, Azo dye, Indoxyl
β-galactosidase	1	Azo dye
β-glucosidase	1	Azo dye
Amine oxidase	3	Aldehyde-Schiff, Tryptamine, Tetrazolium
Dehydrogenases (5)	1	Tetrazolium
Diaphorases (2)	1	Tetrazolium
Carbonic anhydrase	1	Co-S

Since 1939 a tremendous transformation has taken place and methods now exist for twelve enzymes or enzyme groups belonging to the hydrolases. A phosphorylase, three glycosidases, an adding enzyme (carbonic anhydrase), a further oxidase, two flavoprotein enzymes (diaphorases) and a mixed bag of [illegible]
[illegible]

[illegible] designed to demonstrate enzymes in this last group is open to question and there is now

antibody methods, introduced by Coons and his collaborators (Coons, Creech, Jones and Berliner, 1942), have been used successfully by several groups of workers (e.g. Marshall, 1951;

TABLE 3. Enzymes

Before 1900		*Before 1939*	
Peroxidases	Guaiac	Peroxidases	Benzidine and zinc-leuco
Indophenol (Cytochrome) oxidase		Phenoloxidases	α-naphthol
		DOPA-oxidase	Bloch-Laidlaw
		Myrosulphatase	*Peche*

Hill and Cruikshank, 1953; White, 1954). They depend on the production of a specific antibody and on the coupling, *in vitro*, of the ε-NH_2 groups of its constituent lysine molecules with fluorescein isocyanate. The resulting fluorescent antibody complex attaches itself to the antigen in tissue sections (freeze-dried or fresh-frozen), when applied to them, and the sites of attachment are revealed by fluorescence microscopy. At present the application of Coon's particularly elegant technique is beset with numerous problems of a purely technical nature.

With the exception of the fluorescent antibody methods the techniques of protein histochemistry have limited application or, rather, the information derived from their use is of limited value.

Although the number of new carbohydrate techniques has been considerably fewer than those in the protein field one in particular (*the periodic acid-Schiff method of McManus, 1946*) has proved to be of exceptional value. Some of its many applications I propose to illustrate and discuss in the final section. Second in importance to the PAS technique is the use of Alcian blue (Steedman, 1950; Pearse, 1953a) for demonstrating sulphated mucins. The alkaline tetrazolium method (Pearse, 1953b) is also capable of giving useful information about the various mucins, especially when used in conjunction with the above methods.

From Table 3 it is evident that up to the year 1939 the only

It is difficult to make any accurate division in time when considering the physical methods. With most of those employed today the original histochemical use can be traced back to well

TABLE 7. Nucleoproteins

Up to 1945		*After 1945*
Fuelgen	DNA	Perchloric acid extractions
Hydroxyfluorones	DNA, RNA	
MG-Pyronin	DNA, RNA	(Autoradiography)
Ribonuclease	RNA	
Deoxyribonuclease	DNA	
Gallocyanin	DNA, RNA	
NaCl extractions		
Bile salt extractions		
(U.V. Spectrophotometry)		
(Visible Spectrophotometry)		

before 1945. Even interference and electron microscopy, placed on the opposite side of the table, do not properly belong there. There has been, however, a growing number of modern applications of methods employing older or newer principles of microscopy or microspectrophotometry which suggest that in the future tissue histochemistry will come to rely on these more and more for accurate analysis of tissue components.

The great majority of methods for inorganic anions and cations are of some antiquity and, with certain well-known exceptions, their performance is far from satisfactory. Autoradiography, which should more properly be considered among the physical methods, has produced the only striking advances in the localization of inorganic components recorded since 1945.

The most significant feature of Table 7 is the smallness of both columns. This emphasizes how much the histochemistry of the nucleoproteins has relied and still relies on less than a handful of techniques. Modern advances in knowledge have come largely from the applications either of U.V. spectrophoto- [illegible] ^{32}P and ^{35}S.

Most work on tissue lipids is still performed by means of

some uncertainty as to whether the substrate specific dehydrogenases themselves are in fact responsible for the reactions observed in tissue sections. Particular reference to the applications of a number of reliable histochemical methods for enzymes will be made in the final section.

TABLE 5. Physical Methods

Up to 1945	*Since 1945*
Polarization Microscopy	(*Interference Microscopy*)
Fluorescence Microscopy	(*Electron Microscopy*)
Analytical Electron Microscopy	
Visible Spectrophotometry	
U.V. Spectrophotometry	
X-ray Spectrophotometry	
Phase Microscopy	

TABLE 6. Inorganic Constituents

Up to 1945		*After 1945*	
Microincineration	various metals	Autoradiography	various ions
Macallum	K		
Alizarin	Ca	Rhodizonate	Ba and Sr (Ca)
von Kóssa	PO_4		
Crétin	Ca	Naphthochrome	Be
Perls'	Fe		
Quinke	Fe	Calcium red	Ca
Mallory	Cu		
Nitroprusside	Zn	Cu phthalocyanins	Ca, Ba
D.P. thiocarbazide	Zn, Hg		
Stannous chloride	Au		
Iodo-quinine	Bi		
Ammon: sulphide	Pb		
Chromate	Pb		
Ferrocyanide	U		
Titan Yellow	Mg		
Rubeanic acid	Cu		
Rhodanine	Cu		
Sodium ammonium phosphate	Mn		
Silver nitrate	Cl		
Molybdate	PO_4		

It is difficult to make any accurate division in time when considering the physical methods. With most of those employed today the original histochemical use can be traced back to well

TABLE 7. Nucleoproteins

Up to 1945		*After 1945*
Fuelgen	DNA	Perchloric acid extractions
Hydroxyfluorones	DNA, RNA	
MG-*Pyronin*	*DNA, RNA*	*(Autoradiography)*
Ribonuclease	RNA	
Deoxyribonuclease	DNA	
Gallocyanin	DNA, RNA	
NaCl extractions		
Bile salt extractions		
(U.V Spectrophotometry)		
(Visible Spectrophotometry)		

before 1945. Even interference and electron microscopy, placed on the opposite side of the table, do not properly belong there. There has been, however, a growing number of modern applications of methods employing older or newer principles of microscopy or microspectrophotometry which suggest that in the future tissue histochemistry will come to rely on these more and more for accurate analysis of tissue components.

The great majority of methods for inorganic anions and cations are of some antiquity and, with certain well-known exceptions, their performance is far from satisfactory. Autoradiography, which should more properly be considered among the physical methods, has produced the only striking advances in the localization of inorganic components recorded since 1945.

The most significant feature of Table 7 is the smallness of both columns. This emphasizes how much the histochemistry of the nucleo*proteins has relied and still relies on less than a* handful of techniques. Modern advances in knowledge have come largely from the applications either of U.V. spectrophotometry, or visible spectrophotometry applied to the Feulgen and methyl green-pyronin reactions, or from autoradiography with ^{32}P and ^{35}S.

Most work on tissue lipids is still performed by means of

methods which have been in use for a very long time. For the triglycerides, the fatty acids, and for cholesterol, no new methods are in use. In the case of the phospholipids (and the cerebrosides) better fixation has resulted in improved localization even when using the older techniques. This fixation

TABLE 8. Lipids

Before 1945	
Dye Solvent Methods	*Cholesterol Methods*
Red Sudan Dyes	Schultz
Blue BZL	Bismuth Trichloride
Sudan Black B	
Fettrot	*Phosphatide Methods*
Oil Red Dyes	Feyrter
	Alsterberg
2° Fluorescence Methods	Ciaccio
Benzpyrene	Smith-Dietrich
Phosphine	
Rhodamine B	*Other Methods*
	Fischler (fatty acids)
Extraction Methods	Nile Blue Sulphate
Keilig	(neutral and acidic lipids)
	OsO_4 reduction
Polarization Methods	(reducing lipids)
Refringence Tests	Acid fastness
Digitonin Reaction	

TABLE 9. Lipids

After 1945	
Acid Haematein	Phospholipids
Peracetic acid—Schiff	Unsat. Lipids
Performic acid—Schiff	Unsat. Lipids
Phosphomolybdic Acid/$SnCl_2$	Phospholipids
Diphenylcarbazone Methods	Phospholipids
Cu Phthalocyanin Methods	Phospholipids
Tetrazolium Methods	Reducing Lipids

depends on the use of formalin containing calcium ions (Baker, 1944) with or without the addition of cobalt and cadmium. With these metals the phospholipids form complexes called co-acervates which are less diffusible in aqueous solutions (though not usually less soluble in fat solvents) and their pre-

servation is thereby improved. The most specific of the newer methods for phospholipids, Baker's (1946) acid haematein method, is directly derived from the older Smith-Dietrich procedure. Of the other methods, when used in conjunction, the peracetic acid-Schiff method (Pearse, 1951; Lillie, 1952), the

TABLE 10 Aldehydes and Ketones

Before 1945	*After 1945*
Ammoniacal Silver	Phenyl hydrazine-formazan
Nitrohydroxylamine	
Schiff Reaction	Naphthoic acid hydrazide-dianisidine (N.A H D)
Plasmal Reaction	
Phenylhydrazine	
Dinitro-phenylhydrazine	Hydroxylamine (block)
Bisulphite (block)	
Cyanide (block)	
Dimedone (block)	
Semicarbazide (block)	
Sulphanilic acid (block)	

copper phthalocyanin methods (Kluver and Barrera, 1953; Pearse, 1955a), and the alkaline tetrazolium method (Pearse, 1953b) afford some insight into the make-up of tissue phospholipids and, particularly, of their oxidation products the lipid pigments.

Over the histochemical demonstration of ketosteroids, since 1940, there has been great argument concerning the specificity of the various methods for aldehydes on the one hand and for *ketosteroids* on the other (e.g Bennett, 1940; Ashbel and Seligman, 1949; Greep and Deane, 1949; Gomori, 1942, 1950a). With the publication of the recent paper by Karnovsky and Deane (1955), advocates for the specific localization of tissue ketosteroids by any of the current methods may be considered to have withdrawn from the field. It is probable, however, that the application to histochemistry of some of the modern techniques for chromatographic determination of steroids, particularly those depending on the production of fluorescence, will soon provide localization on the histological if not on the cytological scale.

Advances in the histochemistry of pigmented substances have largely been made by using the older techniques in conjunction with some of the newer techniques in the right-hand column of the table and with a few techniques 'borrowed' from the other tables. In this way our knowledge of the lipofuscins and of that peculiar lipid pigment ceroid (Lillie, Daft and Sebrell, 1941) has been considerably advanced. The pigment of formalin-fixed enterochromaffin cells forms the subject of a brief digression in the final section of this lecture.

TABLE 11. Pigments

Up to 1945		*Since 1945*	
Silver methods	Melanins	PAS	Lipofuscins
Ferric ferricyanide	Melanins Lipofuscins	PAAS	Lipofuscins
Long Z N	Lipofuscins		
Bleaching	Melanins	Gibbs reaction	E C. granules
Chromaffin	Adrenalin		
Diazonium	E.C. granules	Dam reaction	Fat peroxides
Indophenol	E.C. granules		
Vulpian	o-Diphenols	Tetrazolium	Reducing lipids
Benzidine	Haematein		
Zinc-leuco	Haemoglobin		
Gmelin	Bilirubin		
Stein	Bilirubin		

COMMENTARY ON TABLES 1 TO 11

Altogether about 195 different methods can be extracted or inferred from the tables presented above and, with those which have been inadvertently or intentionally left out it is evident that there are at least 200 techniques within the compass of the science of microscopic histochemistry. It is fortunate indeed that a number are of historical interest only and that of the remainder a substantial number are virtually useless.

Three groups of methods stand out particularly in this *analysis: (1) Protein methods, (2) Carbohydrate methods, and* (3) Enzyme methods. Reviewing the literature of applied histochemistry however, it is apparent that it is the carbohydrate and enzyme methods which have commanded the most interest in recent years. For this reason, and because some restriction of





Fig. 1. (*left*) Human duodenal mucosa. Goblet cells faintly stained with Alcian blue. (Alcian blue, Neutral red × 300)

Fig. 2 (*right*) Human duodenal mucosa As Fig 1, but pretreated with chlorosulphonic acid in dry pyridine (Alcian blue, Neutral red × 300)



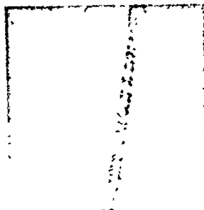


Fig. 3 (*left*) An acontium of *Metridium* in transverse section Below, mucous gland containing a sulphated mucin, above, left fully developed nematocyst staining bright red and, right, developing nematocyst staining blue (Alcian blue, Neutral red × 400)

Fig 4 (*right*) Nematocyst from *Metridium*, longitudinal section The spiral thread alone is stained, indicating that it contains reducing groups (probably derived from chitin) (Alkaline tetrazolium × 1100)

Advances in the histochemistry of pigmented substances have largely been made by using the older techniques in conjunction with some of the newer techniques in the right-hand column of the table and with a few techniques 'borrowed' from the other tables. In this way our knowledge of the lipofuscins and of that peculiar lipid pigment ceroid (Lillie, Daft and Sebrell, 1941) has been considerably advanced. The pigment of formalin-fixed enterochromaffin cells forms the subject of a brief digression in the final section of this lecture.

TABLE 11. Pigments

Up to 1945		*Since 1945*	
Silver methods	Melanins	PAS	Lipofuscins
Ferric ferricyanide	Melanins Lipofuscins	PAAS	Lipofuscins
Long Z N.	*Lipofuscins*		
Bleaching	Melanins	Gibbs reaction	E.C granules
Chromaffin	Adrenalin		
Diazonium	E.C. granules	Dam reaction	Fat peroxides
Indophenol	*E.C. granules*		
Vulpian	*o*-Diphenols	Tetrazolium	Reducing lipids
Benzidine	Haematein		
Zinc-leuco	Haemoglobin		
Gmelin	Bilirubin		
Stein	Bilirubin		

COMMENTARY ON TABLES 1 TO 11

Altogether about 195 different methods can be extracted or inferred from the tables presented above and, with those which have been inadvertently or intentionally left out it is evident that there are at least 200 techniques within the compass of the science of microscopic histochemistry. It is fortunate indeed that a number are of historical interest only and that of the remainder a substantial number are virtually useless.

Three groups of methods stand out particularly in this analysis: (1) Protein methods, (2) Carbohydrate methods, and (3) Enzyme methods. Reviewing the literature of applied histochemistry however, it is apparent that it is the carbohydrate and enzyme methods which have commanded the most interest in recent years. For this reason, and because some restriction of

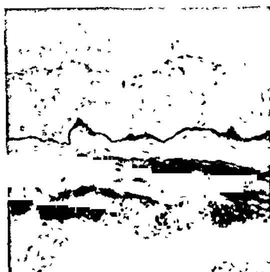


Fig 7 *Eutaenia sirtalis* (Garter snake), stomach Alkaline phosphatase in longitudinal and circular muscle and in muscularis mucosae Incubation time 3 minutes (Coupling azo dye method × 190)



Fig 8 *Photinus pennsylvannica* (Firefly), luminous organ Alkaline phosphatase in epithelium of tracheoles in the outer (granular) layer, and in the surface epithelium Extreme lower right, a strong phosphatase in the intestine (Coupling azo dye method × 400)

PLATE XXVII



FIG. 5. Human kidney, pelvis and collecting tubules From a case of Moniliasis. (Periodic acid-Schiff × 370)

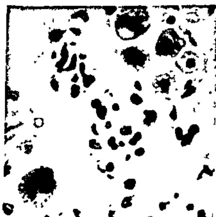


FIG 6. Human lung A group of mucin-containing cells in a lymphatic vessel and, lower left, a dust cell (macrophage) (Periodic acid-Schiff. × 520)



FIG 13. *Buccinum undatum* (whelk). Oocyte. Development of phospholipid granules in an early stage. (Phthalocyanin method, Neutral red × 400)



FIG. 14 Human vermiform appendix In the lamina propria are numerous macrophages containing 'pseudomelanin' pigment. (Schmorl's reaction, Carmalum × 95)

FIG 9 (*left*) *Sceloporus undelatus* (lizard), foregut. Sulphated epithelial mucin (Alcian blue, Neutral red × 65)

FIG 10 (*right*) ... the outer two-thi[illegible] lipids this reactio[illegible] tetrazolium × 27

FIG 11 (*left*) *Buccinum undatum* (whelk) Oocyte Non-specific esterase, indicated by deposits of blue indigo pigment, is restricted to a few circumscribed zones (Indoxyl method, Neutral red × 460)

FIG 12 (*right*) Human kidney from a case of chloroma (myeloid leukaemia) All the myeloid cells contain bright red thioindigo Indicates presence of an 'oxidase' system (4-methyl-6-chlorothionaphthene, Haemalum × 420)



FIG. 13 *Buccinum undatum* (whelk). Oocyte. Development of phospholipid granules in an early stage. (Phthalocyanin method, Neutral red. × 400)

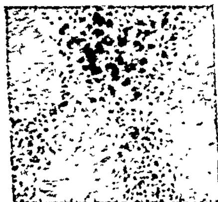


FIG 14 Human vermiform appendix In the lamina propria are numerous macrophages containing 'pseudomelanin' pigment (Schmorl's reaction, Carmalum × 95)

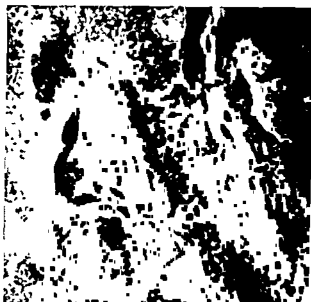


FIG. 15. Human bone. Two groups of dark-staining osteoclasts containing a strong acid phosphatase. In the osteoblasts and osteocytes this enzyme is weaker. (Coupling azo dye method, Methyl green. × 220)



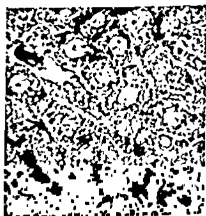


FIG. 16 (*left*). Rat liver. A non-specific esterase is present in groups of irregular-shaped bodies lying at the periphery of the cells, along the bile canaliculi. It is also present in perinuclear bodies (? Golgi region). (Indoxyl method, Neutral red. × 460)

FIG. 17 (*right*). Rat liver. Non-specific esterase by a coupling azo dye method. To show close resemblance to localization indicated by indoxyl method (Fig. 16). (2-acetoxy-3-naphthoic-*o*-toluidide, Methyl green. × 320)

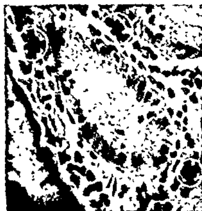


FIG 18 Human ileum. In the lamina propria are three eosinophils whose granules are obscured by a black melanin deposit. (DOPA reaction, pH 6 8, Neutral red. × 300)



FIG 19 Human ileum Three enterochromaffin cells stand out clearly. The formalin-fixed granules in these cells have been oxidized by means of alcoholic iodine and reacted with a thionaphthene compound (Haematum × 300)





FIG 20. (*left*) *Triton rubicundus*. 'Melanoblast' The darkest patches in the epithelium are pre-existing melanin, fainter grey patches are part of a positive reaction. No preformed pigment was present in the 'melanoblast'. (DOPA reaction. × 335)

FIG. 21 (*right*) As Fig 20 Incubated with an indoxyl acetate in the absence of oxidizers The 'melanoblast' contains a large amount of indigo pigment. (× 335)



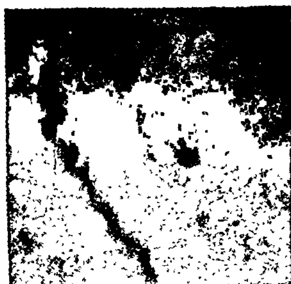


FIG. 23 Rat duodenum Freeze-dried, unfixed Autofluorescence only in lipids of the lamina propria. (× 570)



FIG. 24 As Fig 23, but exposed to formaldehyde vapour for 10 minutes before mounting Autofluorescence of lipids largely quenched, enterochromaffin cell granules now fluoresce bright yellow (× 570)



FIG 20 (*left*) *Triton rubicundus*. 'Melanoblast.' The darkest patches in the epithelium are pre-existing melanin, fainter grey patches are part of a positive reaction No preformed pigment was present in the 'melanoblast'. (DOPA reaction. × 335)

FIG 21. (*right*) As Fig 20. Incubated with an indoxyl acetate in the absence of oxidizers. The 'melanoblast' contains a large amount of indigo pigment (× 335)



FIG 22 Control section for Fig 21 Incubated with an indoxyl acetate in

so large a subject is obviously necessary, the discussions and illustrations of my final section have been largely confined to these two. First, however, it is necessary to consider the broader implications of histochemistry as an applied science.

The Applications of Histochemistry

Histochemical methods have been and can be used in many ways in their application to the basic sciences. In Table 12, below, these different ways have been subdivided into three columns, each independent of the other. In studying the chemical nature and distribution of a (lipofuscin) pigment in the gonads of two different species, or in one species at two different times, the methods are being used in a manner *qualitative*, *analytical* and *comparative*. If we measure the rate of hydrolysis of a particular substrate by the cells of an undifferentiated tumour, using the result to deduce the probable ancestor of these cells, and thus the precise nature of the tumour, then the manner of use is *quantitative*, *functional* and *diagnostic*.

At the present time it is unfortunately true to say that tissue histochemistry is largely a qualitative science. This is because the methods available for measuring substances, natural or artificial, in cells and tissues are complicated and need expensive apparatus not available in most laboratories Furthermore, the physical conditions prevailing in tissue sections are not precisely controllable, as are those in the test-tube. Gross errors may occur, for instance, due to irregular distribution of substances within the cell, which make accurate quantitation impossible.

In considering microscopic quantitative histochemistry we must except the elegant methods of the Linderstrøm-Lang school, depending on the use of alternate fresh-frozen sections (one for histology and the other for microchemistry) and the equally elegant methods of Lowry (1953) who removes and weighs single cells before applying to them various microchemical assays. With both these techniques, for instance, quantitative values for the activity of almost any enzyme may be obtained. Conventional tissue histochemistry, on the other

Interferometric Methods

Interference microscopy has been used for the measurement of section thickness, an essential piece of information when absolute measurement of the quantity of any intracellular substance is required though unnecessary if such measurement is only comparative.

More recently (Davies, Barter and Danielli, 1954; Davies *et al.*, 1955) it has been applied to the quantitative estimation of chemical precipitates deposited on cells by enzyme activity. It may thus provide an alternative, in many cases, to visible or U.V. spectrophotometry.

Semiquantitative Methods

The oldest semiquantitative method, used largely for comparative purposes, is that of cell counting. This, even when a statistically valid method of sampling is used, necessitates the laborious counting of a large number of cells if error is to be reduced to reasonable proportions. Other sampling methods are used which measure the relative areas in tissue sections occupied by the component being studied. The relative volumes are obtained by simple calculation. Line sampling (Uotila and Kannas, 1952) and point sampling (Chalkley, 1943) are examples of this type. Both are fully discussed by Eränko in his book. I do not propose to deal separately with the other headings in the second and third columns of Table 12, which should be self-explanatory, but to conclude by giving some examples of the applications of histochemical methods in three particular groups, the carbohydrate methods, enzyme methods and methods applicable to the study of pigmented substances.

Carbohydrate Methods and their Applications

The main applications of the methods in this group are to the study of those huge classes of substances the mucins (mucopolysaccharide-protein complexes) and mucoids (mucoproteins, polysaccharide-proteins). Attempts have been made (Grishman, 1952, Lennox, Pearse and Richards, 1952; Yates and Paget, 1952) to use modern histochemical methods for the differentiation of mucins from human sources into different

hand, is restricted to the list appearing in Table 4. In measuring the performance of the Purkinje cell of the cerebellum Lowry removes this cell from freeze-dried sections by microdissection. His methods cannot be applied to cells which are not so removable (but only to the tissue layers containing them). With conventional histochemistry the enzyme content of any number of individual cells, up to a limit far in excess of that approachable

TABLE 12. Histochemical Applications

		COMPARATIVE
QUANTITATIVE		
	ANALYTICAL	
SEMIQUANTITATIVE		MORPHOLOGICAL
	FUNCTIONAL	
QUALITATIVE		
		DIAGNOSTIC

by any other means, is readily demonstrable. Difficulties arise only when the measurement of the coloured or colourless end product of this enzyme activity is attempted.

PHOTOMETRIC METHODS

The application of spectrophotometry, using visible or ultraviolet light, has not proved easy and methods employing this principle have usually been criticized more or less severely on physical grounds. The possible error has been variously estimated at figures ranging between 10 and 100 per cent. It is not usually recognized, however, by those to whom mensuration is an exact science, that tissue histochemistry can usefully employ methods giving quite an approximate estimate of quantity—to replace the arbitrary 1–2–3+ system which figures so largely in current researches. In his very acceptable book *Quantitative Methods in Histology and Microscopic Histochemistry* (1955) Eränkö makes this point very clearly. This book offers some encouragement to those histochemists for whom the physical techniques of measurement are mysterious and difficult, and to those who have hesitated to employ them for various reasons.

chitin, but not of chitin itself which has long been thought to be the case. The spirally wound thread of the nematocyst with its terminal dart, however, may well be composed of chitin since it fails to react with the PAS reaction (as does chitin elsewhere; Brunet, 1952) but gives a strong reaction with the alkaline tetrazolium reaction (Pearse, 1953b) as illustrated in Plate XXVI, Figure 4. This reaction is positive not only with carbohydrate-containing materials but also with sulphur-containing (SH, SS) proteins and with reducing lipids and lipoproteins. Neither of these can be shown by other methods to be present in the thread.

The use of the alkaline tetrazolium method in place of older methods for chitin, which 'n'etaient guère commodement realisables à cause de leur brutalité' according to Lison (1953), is shown also in Plate XXIX, Figure 10, where the rigid structure of the ctenidium of *Triton rubicundus* is seen to stain brilliantly. Here also, before making the assumption that the positive reaction is due to chitin, it is necessary to perform a number of complementary reactions to exclude non-carbohydrate reducing substances.

Certainly the periodic acid-Schiff (PAS) reaction has become one of the main supports of modern applied histochemistry. Two of its diagnostic uses in pathology have been illustrated in Plate XXVII, Figures 5 and 6, where, in the first, the hyphae of *Monilia* (in a human renal pelvis) are shown very strongly stained and in the second a small group of mucus-secreting cancer cells is seen in a vessel in the lung. A wide variety of pathogenic fungi, including *Monilia* (*Candida*), *Cryptococcus*, *Blastomyces*, *Histoplasma*, *Sporotrichum*, *Tinea* and *Coccidioides*, are most readily demonstrable by means of the PAS reaction, with or without counterstain. For the demonstration of isolated mucin-secreting cancer cells, in smears from ascitic or pleural fluids, or in small biopsies of liver, omentum, or subcutaneous tissues, the PAS reaction is greatly superior to any other. It finds regular use in diagnostic pathology as a glycogen stain, using always a serial or adjacent section pretreated with diastase as a control. The morphological uses of the PAS reaction include the demonstration of renal glomerular basement membranes

categories. The subject is complicated, however, by the fact that two components have separately to be assessed, the protein component and the sugar component. If the latter is sulphated, as it is in the acid mucopolysaccharides, the choice of methods falls between the older techniques for metachromasia and the newer phthalocyanin method employing Alcian blue (Steedman, 1950). The morphological use of this method is shown in Plate XXIX, Figure 9,[1] where the sulphated mucin of the foregut of the Eastern Fence Swift Lizard (*Sceloporus undelatus*) is clearly demonstrated. The affinity of the water-soluble phthalocyanins of the Alcian blue class for acid groups other than sulphate (PO_4, COOH) presents difficulties, in paraffin sections, only in the occurrence of moderate staining in collagens and similar proteins. This can be avoided (Mowry, 1954) by using very dilute solutions, at pH 2·0 or thereabouts, for twenty-four hours at room temperature. The relative specificity of Alcian blue for the sulphated mucins is shown in Plate XXVI, Figures 1 and 2. In the former, the poorly sulphated mucin of the goblet cells of the duodenum stains feebly blue. After eight hours' treatment with 0·8 per cent chlorosulphonic acid in dry pyridine brilliant staining of the now fully sulphated mucin is produced. Quantitative methods have not been applied to the study of mucins by phthalocyanin and sulphation techniques. The spectral characteristics of these dyes should lend themselves readily to spectrophotometry.

An analytical problem is illustrated by Plate XXVI, Figure 3, which shows in transverse section an acontium of the sea-anemone *Metridium*. It contains mucous glands and is armed with nematocysts, two of which are seen clearly to the left of the mid-line. The capsules of these organs have long been known to have a strong affinity for basic dyes and the nematocyst which reaches the surface of the acontium is deeply stained with the counterstain (neutral red chloride). The other one of the pair is stained only with Alcian blue, as are the mucous glands. Here is the suggestion that in the earlier stages of development the capsule of the nematocyst is composed of an acid mucopolysaccharide, possibly a sulphated

[1] The plates referred to in this lecture will be found between pages 368–9

the latter for use on frozen sections and gives good results on paraffin sections from either cold acetone or cold formalin fixed tissues. With a strong enzyme the incubation period may be as short as five minutes; such a case is illustrated in Plate XXVIII, Figure 7, where the circular and longitudinal smooth muscle, and the muscularis mucosae, in the stomach of the North American Garter Snake (*Eutaenia sirtalis*), are strongly demonstrated with such brief incubation. This is plainly a morphological use but the method could doubtless be used to assess the function of *Eutaenia* smooth muscle much as the so-called succinic dehydrogenase (tetrazolium) methods have been used as indications of function in mammalian cardiac muscle (Wachstein and Meisel, 1954).

During some researches into the possibility of demonstrating adenosine triphosphatase in the luminous organ of the firefly (*Photinus pennsylvanica*), a possibility which remained unrealized, a strong non-specific alkaline phosphatase (α-naphthyl phosphatase) was observed in the respiratory epithelium of the tracheoles. The enzyme was weak or absent from the tracheoles except where these penetrated into the main granular layer of cells, and travelled through them. These appearances are illustrated in Plate XXVIII, Figure 8. The presence of so strong an enzyme in just those regions where the passage of O_2 and CO_2 across the membrane must at times reach high levels is surely significant of some higher activity such as energy production, but our knowledge of the actual function of the alkaline phosphatase, within and without the cell, is not sufficiently adequate to allow more than the interesting speculation. The hypothesis of Danielli (1953) that they are associated with contractile proteins scarcely seems applicable in this case

In the last few years many efforts have been made (Goetsch and Reynolds, 1951; Seligman and Manheimer, 1949; Grogg and Pearse, 1952, Burton, 1954) to modify or replace the original (Gomori, 1941) method for acid phosphatases. These have not been particularly successful but some of the coupling azo dye techniques have at least improved the accuracy with which the enzyme can be localized. In Plate XXXI, Figure 15, appear two small groups of osteoclasts which are engaged in removing

and the mucoprotein-secreting cells (basophils) of the anterior pituitary gland.

In all comparative studies of mucin the PAS method now takes precedence over the rest and forms the cornerstone on which the results of the other tests can be built up. Differences in the degree of staining obtained in identical structures by different authors are certainly largely due first, to fixation differences and secondly, to variations in the mode of application of the method. This last point has been well-stressed by McManus (1954) in pointing out the necessity always to distinguish between the use of periodic acid followed by the reducing rinse of Hotchkiss (1948) before treatment with Schiff's reagent, and its use without the rinse. McManus suggests the term PARS for the first and PAS only for the second. Using the PAS, PARS, Alcian blue and alkaline tetrazolium methods, together with the Gram reaction and methylene blue extinction, metachromasia and sulphation, and extraction with hyaluronidases, pectinases and alkali, pertinent inquiry can be made into the nature of any mucin or mucoprotein found in the tissues. Advances in our knowledge of the mucins and their function are to be expected from the further application of these techniques.

Enzyme Methods and their Application

Alkaline and acid phosphatases

Between 1939 and 1953 probably more work was published on the application of the alkaline phosphatase method (Gomori's original method using glycerophosphate as substrate and the calcium-cobalt or calcium-silver methods for trapping the free phosphate ions and subsequently making them visible as brown or black precipitates) than on the application of any other single method. The method itself has survived a great deal of critical work on the accuracy of localization of the enzyme but there has recently been some falling off in the number of problems to which it has been applied. The coupling azo dye method of Menten, Junge and Green (1944), modified by many groups of workers, has risen in popularity but has never achieved the success of the original Gomori method. It is certainly superior to

the latter for use on frozen sections and gives good results on paraffin sections from either cold acetone or cold formalin fixed tissues. With a strong enzyme the incubation period may be as short as five minutes; such a case is illustrated in Plate XXVIII, Figure 7, where the circular and longitudinal smooth muscle, and the muscularis mucosae, in the stomach of the North American Garter Snake (*Eutaenia sirtalis*), are strongly demonstrated with such brief incubation. This is plainly a morphological use but the method could doubtless be used to assess the function of *Eutaenia* smooth muscle much as the so-called succinic dehydrogenase (tetrazolium) methods have been used as indications of function in mammalian cardiac muscle (Wachstein and Meisel, 1954).

During some researches into the possibility of demonstrating adenosine triphosphatase in the luminous organ of the firefly (*Photinus pennsylvanica*), a possibility which remained unrealized, a strong non-specific alkaline phosphatase (α-naphthyl phosphatase) was observed in the respiratory epithelium of the tracheoles. The enzyme was weak or absent from the tracheoles except where these penetrated into the main granular layer of cells, and travelled through them. These appearances are illustrated in Plate XXVIII, Figure 8 The presence of so strong an enzyme in just those regions where the passage of O_2 and CO_2 across the membrane must at times reach high levels is surely significant of some higher activity such as energy production, but our knowledge of the actual function of the alkaline phosphatase, within and without the cell, is not sufficiently adequate

and Reynolds, 1951; Seligman and Manheimer, 1949; Grogg and Pearse, 1952; Burton, 1954) to modify or replace the original (Gomori, 1941) method for acid phosphatases. These have not been particularly successful but some of the coupling azo dye techniques have at least improved the accuracy with which the enzyme can be localized. In Plate XXXI, Figure 15, appear two small groups of osteoclasts which are engaged in removing

the bone trabeculae on which they lie. With a simultaneous coupling technique the strong acid phosphatase which they contain can be demonstrated with a very short period of incubation, cutting diffusion of the reaction products to a minimum. This diffusion prevents the use of the method with such prolonged incubation times as are necessary for the majority of (weak) acid phosphatases present in mammalian tissues. Acid phosphatase is not confined to the osteoclasts among phagocytic cells. In the majority of mammals the macrophages (and the monocytes from which many of them are derived) have a similar enzyme content. This may perhaps be correlated with the fact that the intracytoplasmic reaction of such cells, in the mouse and rat, appears to be acid (Rous, 1925). The probable direction in which the azo dye techniques for acid phosphatase are likely to proceed is suggested by the newest method of Rutenberg and Seligman (1954) using a post-incubation coupling technique and 6-benzoyl-2-naphthyl phosphate as the substrate.

Non-specific Esterases

A variety of reliable histochemical techniques now exist which demonstrate members of the broad group of more or less closely related enzymes called non-specific esterases. Interest in these techniques and in their applications has risen sharply in the last few years but we are still a long way from understanding the particular functions which these enzymes perform in the cells which contain them. The most reliable and most commonly employed method is the so-called α-naphthyl esterase method of Gomori (1950b) which uses the easily obtainable α-naphthyl acetate as substrate. Intracellular localization of enzyme by this method is inaccurate because of diffusion of the primary reaction product (α-naphthol) and because of the solubility of the final azo dye product in lipids. With the coming of the indoxyl acetate methods, using the halogen-substituted indoxyl esters introduced by Holt (1952), a far greater sharpness of intracellular localization has been achieved. In a variety of cells from mammalian and other sources non-specific esterase has been shown to be distributed not throughout the cytoplasm

(whether in microsomes, mitochondria or cell sap) but in discrete organelles. In the oocytes of the marine gastropod *Buccinum*, illustrated in Plate XXIX, Figure 11, the final blue deposit of 5-5'-dibromindigo is localized in a single principal and sometimes in one, or two, secondary areas in the cytoplasm. These regions may contain components of the Golgi apparatus but a more attractive hypothesis is that they represent the centrioles or, properly, the regions about them (centrosomes). In any case further work of a comparative nature, using preferably the cells of many widely separated species, should be capable of elucidating this particular problem.

In the liver of the rat the distribution of esterase shown by the indoxyl acetate method (Plate XXXI, Figure 16) differs considerably from that revealed by other methods. The enzyme appears to be concentrated mainly at the free borders of the cells, in bodies of assorted size and shape, thus seeming to outline the bile canaliculi. In some cells an additional larger and usually rounded focus occurs in the perinuclear region, as in the middle cell at the upper border of the illustration. This again may be equated with the Golgi region or perhaps even with the centrosome.

It is possible to achieve a similar localization using the entirely different coupling azo dye principle, provided that a substituted naphthyl acetate yielding a very insoluble primary reaction product is used and provided that the diazonium salt (which couples with it to provide the final azo dye product) is present in the incubating medium. In Plate XXXI, Figure 17, the substrate has been 2-acetoxy-3-naphthoic-*o*-toluidide (Pearse, 1954a) and the final precipitate can be observed to resemble closely that given by the indoxyl method. The close correspondence between the pictures produced by these essentially different methods gives grounds for the belief that one, at least, of the closely related family of esterases present in rat liver cells is actually localized in the sites indicated.

The absence of any yardstick, other than the results of enzyme assay on the various fractions of ultracentrifugated homogenates, makes interpretation of the finer intracellular localizations of any enzyme, as shown by histochemical tech-

niques, a difficult problem indeed. If a number of methods, based on entirely different principles, give an identical localization this is strong presumptive evidence for its accuracy.

Oxidases

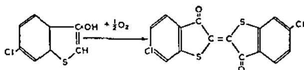
The historical methods of enzyme histochemistry, as a glance at Table 3 will show, were nearly all for oxidases and the most reliable and informative of these methods was undoubtedly the DOPA-oxidase method of Bloch (1917). Within recent years, however, the identity of the enzyme responsible for the DOPA reaction in tissue sections has become less clear. The extreme lability of the enzyme observed by Bloch has been shown by Van Duijn (1953) to have been due to its inactivation by removal and not to inhibition *per se*, and there has recently been some attempt to link up the histochemical DOPA-oxidase with the relatively stable copper-containing enzyme tyrosinase. Van Duijn, however, concludes that present data do not permit such a conclusion although it remains a possibility.

These opinions do not prevent the method from being used in functional studies such as those made by Vercauteren (1951) who believes that the strong 'DOPA-oxidase' present in horse eosinophils is actually a histaminase. In human tissues, as shown in Plate XXXII, Figure 18, the eosinophils sometimes give a similar reaction. In many tissues it is probable that the DOPA method reveals a non-specific phenol oxidase capable of acting on a number of substrates.

In the loose sub-epithelial connective tissues below the pigmented parts of the epithelium of the foot in *Triton rubicundus*, and other marine gastropods, there are large non-pigmented cells whose processes reach out to penetrate the epithelium. They might reasonably be considered as melanoblasts if they contained DOPA-oxidase, as in fact (Plate XXXIII, Figure 20) they do. But Plate XXXIII, Figure 21, shows that they also contain a strong non-specific oxidase which is probably a phenol oxidase. This picture was produced by incubating cold-formalin fixed frozen sections with an indoxyl acetate at pH 8·0 (in the absence of any exogenous oxidizing agent). The colour (black) in the epithelium is entirely due to preformed melanin, only the

'melanoblasts' were stained deep blue. That the enzyme present is one which can convert indoxyl esters to indigo in the presence of molecular oxygen is shown in Plate XXXIII, Figure 22, prepared under identical conditions to Figure 21 but with the addition of an exogenous oxidation catalyst (0·02 M potassium ferricyanide). Here the 'melanoblasts' show no reaction and are therefore free from esterase (the ferricyanide completely inhibits the oxidase), but in the non-pigmented epithelium there is now shown a moderately strong esterase, previously not visible.

These results suggested the possibility, independently confirmed *in vitro* by biochemical studies, that indoxyl derivatives might be useful in histochemistry not only for the demonstration of hydrolases but also for a variety of oxidizing systems. Free indoxyls are rapidly oxidized to indigos in the presence of oxygen, without the intervention of enzyme or catalyst, but the thioindoxyls are relatively stable.



Some of the latter group of compounds were therefore investigated (Pearse, 1954b) as hydrogen donors in 'oxidase' systems.

In the absence of any exogenous oxidizing agent the myeloid granules of fresh, or formalin-fixed, neutrophil leucocytes were observed to bring about the oxidation of suitable free thioindoxyls (thionaphthenes) to red or brown thioindigos (Plate XXIX, Figure 12), but the mechanism by which they do this is still far from clear. Recent revival in histochemical interest in the 'oxidases', particularly those of the leucocytes, is shown by papers by Lillie and Burtner (1953) and Gomori (1953). The second author has clearly shown that the older α-naphthol and M-Nadi (stable indophenol oxidase) reactions of the myeloid granules are not due to enzyme activity but that the more commonly used benzidine reaction shows a perioxidase-like effect, perhaps enzymatic, perhaps due to metal catalysis (Iron or Copper).

Copper (Cu^{++}), in particular, is able to catalyse the oxidation

of indoxyls and thioindoxyls to the corresponding indigos but efforts (Pearse, unpublished) to demonstrate copper in the livers, kidneys and choroids from cases of Kinnier-Wilson's disease (hepato-lenticular degeneration) have proved almost completely unsuccessful. The critical factor here is the state in which copper occurs in animal tissues; it is present in an *unreactive form which must be broken down before activity* due to the cupric ion can be demonstrated.

The time is certainly ripe for a renewed onslaught on the oxidizing systems of various tissues and consideration should be given to the indoxyl and thioindoxyl methods in view of the excellent localization which they are capable of affording.

Lipids, Lipofuscins and Pigments

Of the newer methods for lipids shown in Table 9 I should like to single out one in particular for special mention. This is the Copper Phthalocyanin method (Kluver and Barrera, 1953; Pearse, 1955a). The original authors used Luxol Fast Blue MBS (DuPont) as a stain for myelin without investigating the nature of the reaction in any way. This dye is the alcohol- [illegible]

degree only, with other tissue components. In the developing [illegible] 13) large lipid [illegible] the whole cell.

The chemical and physical properties of the phthalocyanin dyes, many of which are convertible by mild treatment with acids or alkalis into completely insoluble deep blue pigments, make them particularly suitable for histochemistry in the qualitative field and their widespread application to lipid and lipoprotein problems in many of the basic sciences may be expected to yield useful information.

Schmorl's ferric ferricyanide reaction, illustrated in Plate XXX, Figure 14, is an old reaction introduced first in 1909 by Golodetz and Unna who showed that many tissue components,

including unsaturated fatty acids (in combination) were able to reduce ferricyanide to ferrocyanide and thus bring about the production of Prussian blue in the presence of a ferric salt. In modern histochemistry it proves particularly useful in the way indicated by Schmorl (1928) as an indicator of lipofuscin pigments containing reducing groups, presumably aldehydes derived from autoxidation of the original lipid. There is a considerable degree of overlap between this method and the alkaline tetrazolium reaction but if the second is first performed on a section, photographed, and then reversed by removal of the formazan dye in alcohol/xylene before Schmorl's reaction is applied, appreciable differences are observed. The nature of these remains to be explained. Meanwhile, there are a sufficient number of reliable and reproducible methods for phospholipids and lipid pigments to enable the nature and type of any *observed component in these classes to be delineated by the* observer so that workers in other establishments and countries may make comparison with their own material. This particular use of histochemistry, though not spectacular or immediately rewarding, is nevertheless of considerable importance, and workers in the basic sciences cannot be too strongly recommended to use the available methods of histochemistry in this way.

The composition of many of the pigmented substances in the tissues is fairly well established but upon that of others there is considerable disagreement. The so-called pseudo-melanin of the intestinal lamina propria (Plate XXX, Figure 14) remains of uncertain origin and composition as does the yellow pigment of the intestinal enterochromaffin cells, which are widely distributed throughout the animal kingdom. Beginning with the researches of Cordier (1927) and Cordier and Lison (1930), histochemists, until 1948, were content to interpret a long series of positive reactions given by the specific granules of the cells as indicating the presence of an *o*-diphenol (catechol) with a short substituent in one of the *p*-positions. In that year Gomori produced strong evidence that the granules contained a *m*-diphenol (resorcinol) rather than a catechol. Meanwhile, the pharmacological, and to a lesser extent histochemical, researches of Erspamer and his colleagues (e.g. Erspamer and

Asero, 1952) resulted in the hypothesis that the specific component of the granules is 5-hydroxytryptamine. Investigating this particular disagreement Barter and Pearse (1953, 1955) found that the characteristic fluorescence of the enterochromaffin cells, absent in freeze-dried sections, could be produced by as little as ten minutes' exposure of such sections to formalin vapour. Plate XXXIV, Figure 23, shows unfixed, freeze-dried, rat duodenum mounted in nonane and exposed to ultraviolet light. Fluorescence is confined to certain (lipid) materials in the lamina propria. After short exposure to formalin vapour (Plate XXXIV, Figure 24) the lipid fluorescence is quenched, but the enterochromaffin cells show up by their bright yellow colour. Since they were unable to show fluorescence in 5-hydroxytryptamine unexposed to formaldehyde, or in resorcinol derivatives after such exposure, Barter and Pearse concluded that 5-hydroxytryptamine could be and that resorcinols could not be the active substance of the cells. Further work (Pearse, 1955b) has added support to this conclusion. If the formalin-fixed enterochromaffin cell granules are treated with mild oxidizing agents (I_2, IO_3) they will subsequently combine with thioindoxyls at alkaline pH levels to form brownish or blackish dyes, the colour of which closely matches that observed *in vitro* when the bright yellow, strongly fluorescent, 5-hydroxytryptamine-formaldehyde compound is treated in a similar manner. Resorcinol and its derivatives will not react in this way. Plate XXXII, Figure 19, shows the enterochromaffin cells of human duodenum, fixed in formalin, briefly oxidized with 5 per cent alcoholic iodine and coupled with 4-methyl-6-chlorothionaphthene at pH 8.0. The dark brown dye produced shows up clearly in the photograph. Once again, absolute evidence for the presence of 5-hydroxytryptamine in the enterochromaffin cells has not been produced but the evidence available strongly favours this presumption. In this way analytical histochemistry has been used to provide evidence for functional hypotheses.

Conclusions

It has obviously been impossible, in a single lecture, to do more than touch upon the problems to which presently available

histochemical methods can be applied. The few examples I have chosen to illustrate have included several functional applications, together with a few methods widely applicable to inquiry into the state of activity of cells and tissues, and a greater number of qualitative analytical methods with morphological, diagnostic and comparative uses.

At every point of application the simplest selection of histochemical tests produces new and interesting facts and these alone should amply repay the histochemist for his labours. The originally botanical science of histochemistry has become increasingly a histopathological one. Yet, after a few irruptions into the unfamiliar provinces of entomology and marine zoology (some of which feature in this lecture) I am convinced of two things. First, that many fascinating fields of histochemical inquiry exist outside the mammalian species and second, that comparative histochemistry, the study of one animal against another and one tissue against another, and one cell against another, must play an increasingly important role in the future. It offers us some degree of control against too facile speculations based on observation of a single species at a single time, and some real hope of elucidating the pressing problems of interpretation which beset us. What function, for instance, has this esterase in this cell? How can we attempt to answer without making comparison with other esterases, and with other cells, in other animals and in other species.

In conclusion I want to express my belief that there are few problems, particularly in the descriptive sciences, to which histochemical methods cannot be applied with advantage. As I have said before, in other places, it is possible, or even probable, that histochemical methods will not supply the precise answer to any given question, particularly if the question itself is wrongly framed, but a mass of new facts will certainly emerge and out of these, as with bricks, can be built the theories and problems of the future.

ACKNOWLEDGEMENTS

Much of the work mentioned in this lecture was done as a Fulbright Fellow and Visiting Professor of Pathology in the University of

Alabama, and also at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine.

I am greatly indebted to Dr. J. F. A. McManus, M.D., for making such work possible and to Miss Angelina Giovino and Miss Eunice Johnson for their technical assistance.

The illustrations are the work of many hands; I should like to thank Mrs. Gladys MacQueen, Mr. E. V. Wilmott, Mr. C. A. P. Graham and Miss R. Klein for their help in this matter.

REFERENCES

ASHBEL, R. and SELIGMAN, A. M. (1949). *Endocrinology*, **44**, 565.
BAKER, J. R. (1944). *Quart. J. micr. Sci.* **85**, 1.
BAKER, J. R. (1946). *Quart. J. micr. Sci.* **87**, 441.

(1942). *J. Immunol.* **45**, 159
CORDIER, R. (1927) *Bull. Histol. Tech micr* **4**, 161.
CORDIER, R and LISON, L. (1930). *Bull. Histol Tech. micr* **7**, 140.
DANIELLI, J F. (1953) *Cytochemistry*, pp. 75, 76 Chapman and Hall, London.

sto-chemistry Karger, Basle.
ERSPAMER, V. and ASERO, B (1952) *Nature*, **169**, 800.

149.

GOMORI, G. (1950a). *Ann N Y Acad Sci* **50**, 968
GOMORI, G. (1950b) *J. Lab. clin. Med.* **35**, 802.
GOMORI, G. (1952). *Microscopic Histochemistry*. Chicago University Press.
GOMORI, G (1953) *J. Histochem. Cytochem* **1**, 493.
GREEP, R. O. and DEANE, H W. (1949). *Ann N Y Acad. Sci.* **50**, 596.

GRISHMAN, E. (1952) *Cancer*, 5, 700.
GROGG, E and PEARSE, A. G. E. (1952). *J. Path. Bact.* 64, 627.
HILL, A. G. S and CRUIKSHANK, B. (1953). *Brit J exp. Path.* 34, 27.
HOLT, S J (1952). *Nature*, 169, 271.
[illegible]
[illegible], 85.
[illegible]
[illegible] *Path Bact* 64, 865.
LILLIE, R D (1952). *Stain Tech* 27, 37.
LILLIE, R. D and BURTNER, H J. (1953). *J. Histochem Cytochem* 1, 8.
LILLIE, R. D., DAFT, F. S. and SEBRELL, W. H. J. (1941). *Publ. Hlth. Rep. Wash* 56, 1255
[illegible] 31,
ed G. Asboe-Hansen. Munksgaard, Copenhagen.
MENTEN, M. L., JUNGE, J and GREEN, M H. (1944). *J biol Chem.* 153, 471.
[illegible] J and A. Churchill, London
PEARSE, A. G. E. (1953b) *J. Path. Bact.* 67, 129.
PEARSE, A G. E. (1954a). *Int Rev. Cytol.* 3, 329
PEARSE, A G E (1954b). *J. Histochem Cytochem* (Proc Histochem Soc.), 2, 475
PEARSE, A G E. (1955a) *J. Path. Bact* 70, 554
PEARSE, A. G. E (1955b). *Riv Istochimica*, in press.
RASPAIL, F. V. (1830). *Essai de Chimie Microscopique Appliquée à la Physiologie* Paris.
RASPAIL, F V. (1833). *Nouveau Système de Chimie Organique* Paris
ROUS, P. (1925) *J exp Med* 41, 399
RUTENBURG, A. M and SELIGMAN, A. M (1954) *J. Histochem Cytochem* (Proc. Histochem Soc), 2, 445.
SCHMORL, G. (1928) *Pathologisch-Histologischen Untersuchungen Methoden.* Vogel, Leipzig
SELIGMAN, A M and MANHEIMER, L H (1949). *J. nat Cancer Inst* 9, 427
[illegible]

du Vigneaud, V., Ressler, C. and Trippet, S. (1953). *J. biol. Chem.* 205 949.
Wachstein, M. and Meisel, E. (1954). *J. Histochem. Cytochem.* 2, 137.
[illegible] 75, 365
[illegible] *J. Lab. clin. Med.* 41, 296.
[illegible] *Bath. Pact.* 64, 881.

COMPLETE LIST OF LECTURES

** Lectures included in the published volumes*

1951–52

*The Scientific Approach to Medical Research
PROFESSOR E. ADRIAN, O.M., M.D., F.R.C.P., F.R.S. (*Cambridge*)

The Philosophy of Science
PROFESSOR H. DINGLE, D.SC., A.R.C.S. (*London, University College*)

Studies by X-ray Analysis, Electron Microscopy and Supporting Techniques of the Structure of Biological Macromolecules and the Tissues formed from them
PROFESSOR W. T. ASTBURY, M.A., SC.D., F.R.S. (*Leeds*)

*Tissue Responses to Injury
PROFESSOR G. R. CAMERON, M.B., B.S., D.SC., F.R.C.P., F.R.S. (*London, University College Hospital Medical School*)

*Studies of Normal and Pathological Physiology of the Kidney
D. VAN SLYKE, M.D. (*New York, U.S.A.*)

Biochemical Function of Vitamin B_1
PROFESSOR R. A. PETERS, M.D., F.R.S. (*Oxford*)

Nutritional Science and the Feeding of Populations
PROFESSOR S. J. COWELL, M.B., F.R.C.P. (*London, University College Hospital Medical School*)

*Nutritional Assessment of the Individual
PROFESSOR J. YUDKIN, M.A., M.D., PH.D. (*London, Queen Elizabeth College*)

Folic Acid and Vitamin B_{12} in the Metabolism of Micro-organisms
D. D. WOODS, M.A., PH.D. (*Oxford*)

du Vigneaud, V., Ressler, C. and Trippet, S. (1953). *J. biol Chem.* 205 949.
Wachstein, M. and Meisel, E. (1954). *J. Histochem. Cytochem* 2, 137.
White, R. G. (1954). *Brit. J. exp. Path* 35, 365.
Yasuma, A. and Itchikawa, T. (1953). *J. Lab. clin. Med.* 41, 296
Yates, P. O. and Paget, G. E. (1952). *J. Bath. Pact.* 64, 881.

*The Physiological Basis of Visual Sensation
L. C. THOMSON, PH.D. (*London, Institute of Ophthalmology*)

*The Contribution of Science to the Practice of Health in the First Quarter of the Twentieth Century
PROFESSOR J. M. MACKINTOSH, M.D., F.R.C.P., LL.D.
(*London School of Hygiene and Tropical Medicine*)

Methods of Research in Social Medicine
ALICE M. STEWART, M.A., M.D., F.R.C.P. (*Oxford*)

Environmental Factors in Work
PROFESSOR G. P. CROWDEN, O.B.E., T.D., D.SC.
(*London School of Hygiene and Tropical Medicine*)

Methods of Research in Industrial Medicine
DONALD HUNTER, M.D., F.R.C.P. (*London Hospital Medical College*)

*Recent Light on Mammary Function
PROFESSOR H. D. KAY, C.B.E., D.SC., F.R.S. (*Reading*)

*Research on Ageing
PROFESSOR E. C. DODDS, M.D., F.R.C.P., F.R.S.
(*London, Middlesex Hospital Medical School*)

Carcinogenesis
PROFESSOR A. HADDOW, M.D., D.SC., PH.D.
(*London, Institute of Cancer Research*)

Physical Techniques in the Medical Applications of Ionizing Radiations
PROFESSOR W. V. MAYNEORD, D.SC.
(*London, Institute of Cancer Research*)

*Biological Effects of Radiation
J. F. LOUTIT, D.M., M.R.C.P. (*M.R.C., Harwell*)

*The Effects of Haemorrhage on the Cardiovascular System in Man
O. G. EDHOLM, B.SC., M.B., B.S.
(*National Institute for Medical Research, Mill Hill*)

*Lability of Blood Pressure
PROFESSOR M. L. ROSENHEIM, M.A., M.D., F.R.C.P.
(*London, University College Hospital Medical School*)

Aspects of Bacteriostasis
PROFESSOR B. C. J. G. KNIGHT, D.SC. (*Reading*)

*Some Aspects of Antibacterial Immunity
A. A. MILES, M.A., M.B., F.R.C.P.
(*National Institute for Medical Research, Mill Hill*)

*Antibodies and Immunity to Virus Infection
PROFESSOR A. W. DOWNIE, M.D., D.SC. (*Liverpool*)

Current Knowledge on Nature of Viruses
F. C. BAWDEN, M.A., F.R.S. (*Rothamsted Experimental Station*)

**Viruses as the Causes of Diseases*
PROFESSOR S. P. BEDSON, M.D., D.SC., F.R.C.P., F.R.S.
(*London Hospital Medical College*)

*Adrenal Hormones and ACTH
PROFESSOR F. G. YOUNG, M.A., D.SC., F.R.I.C., F.R.S. (*Cambridge*)

Chemical Aspect of Antibiotics
SIR ROBERT ROBINSON, O.M., D.SC., LL.D., F.R.S. (*Oxford*)

*Growth of the Human at the Time of Adolescence
J. M. TANNER, M.D. (*London, St Thomas's Hospital Medical School*)

Bone Growth and Repair
PROFESSOR W. D. NEWCOMB, M.D., F.R.C.P.
(*London, St. Mary's Hospital Medical School*)

The Rate and Materials of Blood Formation
PROFESSOR L. J. WITTS, D.M., F.R.C.P. (*Oxford*)

Alimentary Factors in Blood Formation
PROFESSOR L. J. WITTS, D.M., F.R.C.P. (*Oxford*)

**Blood Coagulation in Theory and Practice*
R. G. MACFARLANE, M.D. (*Oxford*)

The Psychology of Personality
PROFESSOR SIR CYRIL BURT, M.A., D.SC. (*London, University College*)

*On the Organization of Cortical Mechanisms
PROFESSOR SIR GEOFFREY JEFFERSON, M.S., F.R.C.P., F.R.C.S., F.R.S.
(*Manchester*)

Involuntary Nervous System (*2 lectures*)
PROFESSOR G. L. BROWN, C.B.E., M.SC., F.R.S.
(*London, University College*)

*The Physiological Basis of Visual Sensation
L. C. Thomson, Ph.D. (*London, Institute of Ophthalmology*)

*The Contribution of Science to the Practice of Health in the First Quarter of the Twentieth Century
Professor J. M. Mackintosh, M.D., F.R.C.P., LL.D.
(*London School of Hygiene and Tropical Medicine*)

Methods of Research in Social Medicine
Alice M. Stewart, M.A., M.D., F.R.C.P. (*Oxford*)

Environmental Factors in Work
Professor G. P. Crowden, O.B.E., T.D., D.Sc.
(*London School of Hygiene and Tropical Medicine*)

Methods of Research in Industrial Medicine
Donald Hunter, M.D., F.R.C.P. (*London Hospital Medical College*)

*Recent Light on Mammary Function
Professor H. D. Kay, C.B.E., D.Sc., F.R.S. (*Reading*)

*Research on Ageing
Professor E. C. Dodds, M.D., F.R.C.P., F.R.S.
(*London, Middlesex Hospital Medical School*)

Carcinogenesis
Professor A. Haddow, M.D., D.Sc., Ph.D.
(*London, Institute of Cancer Research*)

Physical Techniques in the Medical Applications of Ionizing Radiations
Professor W. V. Mayneord, D.Sc.
(*London, Institute of Cancer Research*)

*Biological Effects of Radiation
J. F. Loutit, D.M., M.R.C.P. (*M.R.C., Harwell*)

*The Effects of Haemorrhage on the Cardiovascular System in Man
O. G. Edholm, B.Sc., M.B., B.S.
(*National Institute for Medical Research, Mill Hill*)

*Lability of Blood Pressure
Professor M. L. Rosenheim, M.A., M.D., F.R.C.P.
(*London, University College Hospital Medical School*)

1952-53

The Influence of Language on Medicine
PROFESSOR J. Z. YOUNG, M.A., F.R.S. *(London, University College)*

The Nature and Place of Thinking in Medicine
PROFESSOR SIR FREDERICK BARTLETT, C.B.E., M.A., F.R.S. *(Cambridge)*

*The Methodology of Clinical Science
PROFESSOR SIR JAMES SPENCE, M.C., M.D., F.R.C.P. *(Newcastle)*

The Statistical Approach
PROFESSOR BRADFORD HILL, C.B.E., D.SC., PH.D. *(London School of Hygiene and Tropical Medicine)*

Control of Respiration
PROFESSOR C. G. DOUGLAS, C.M.G., M.C., M.A., M.D., F.R.S. *(Oxford)*

Life at High Altitudes
PROFESSOR SIR B. H. C. MATTHEWS, C.B.E., M.A., SC.D., F.R.S. *(Cambridge)*

*The Physiological Effects of Gravity
GROUP CAPTAIN W. K. STEWART, C.B.E., A.F.C., R.A.F. *(Farnborough)*

The Kinetics of Rapid Chemical and Biological Reactions
PROFESSOR F. J. W. ROUGHTON, M.A., PH.D., F.R.S. *(Cambridge)*

Metal Catalysis and Intracellular Respiration
PROFESSOR D. KEILIN, F.R.S. *(Cambridge)*

Biophysical Studies of Connective Tissue
PROFESSOR J. T. RANDALL, D.SC., F.R.S. *(London, King's College)*

*The Functional Significance of Connective Tissue
A. H. T. ROBB-SMITH, M.A., M.D., F.R.C.P. *(Oxford)*

*Silicosis
PROFESSOR E. J. KING, PH.D., D.SC. *(Postgraduate Medical School of London)*

Pulmonary Asbestosis
PROFESSOR MATTHEW STEWART, C.B.E., F.R.C.P. *(Leeds)*

Organ Culture in Biological and Medical Research
HONOR B. FELL, F.R.S. *(Cambridge)*

*The Life History of the Malaria Parasite
PROFESSOR P. C. C. GARNHAM, M.D., D.SC., D.P.H.
(London School of Hygiene and Tropical Medicine)

*Human Haemoglobins
J. C. WHITE, M.B., CH.B. *(Postgraduate Medical School of London)*

Uses and Applications of Human Blood Plasma Fractions
SIR ALAN DRURY, C.B.E., M.D., F.R.S *(Cambridge)*

*Preservation of Living Cells at Low Temperatures
A. S. PARKES, M.A., SC.D., F R.S.
(National Institute for Medical Research, Mill Hill)

*The Life-span of Red Blood-cells
P. L. MOLLISON, B.A., M D *(Postgraduate Medical School of London)*

*Biochemical Genetics
A NEUBERGER, PH D , M.D., F.R.S
(National Institute for Medical Research, Mill Hill)

*Chromatography in the Study of Amino-Acid Metabolism
C. E DENT, B SC , PH.D , M D , F R I C , M.R.C.P.
(London, University College Hospital Medical School)

*Selective Toxicity (*2 lectures*)
PROFESSOR ADRIAN ALBERT, D.SC , PH.D., F.R.I.C.
(Australian National University)

*Recent Progress in Antibiotics
PROFESSOR SIR ALEXANDER FLEMING, M.B , B.S., F.R.C.S., F.R.C.P , F.R.S. *(London, St. Mary's Hospital Medical School)*

*Virus Adaptability in Relation to Human Disease
PROFESSOR WILSON SMITH, M D., F R S.
(London, University College Hospital Medical School)

*Carcinogenesis
P. R. PEACOCK, M B., B S., F R.F.P S.G. *(Glasgow)*

*The Metabolism of the Adrenocortical Hormones
PROFESSOR G F. MARRIAN, D SC., F R S *(Edinburgh)*

The Pituitary Gland and Reproduction
S. J. FOLLEY, D.SC., PH.D., F.R.S. *(Reading)*

*The Physiology of Parturition
PROFESSOR A ST G HUGGETT, D SC., PH.D
(London, St. Mary's Hospital Medical School)

1952-53

The Influence of Language on Medicine
PROFESSOR J. Z. YOUNG, M.A., F.R.S. *(London, University College)*

The Nature and Place of Thinking in Medicine
PROFESSOR SIR FREDERICK BARTLETT, C.B.E., M.A., F.R.S. *(Cambridge)*

*The Methodology of Clinical Science
PROFESSOR SIR JAMES SPENCE, M.C., M.D., F.R.C.P. *(Newcastle)*

The Statistical Approach
PROFESSOR BRADFORD HILL, C.B.E., D.SC., PH.D. *(London School of Hygiene and Tropical Medicine)*

Control of Respiration
PROFESSOR C. G. DOUGLAS, C.M.G., M.C., M.A., M.D., F.R.S. *(Oxford)*

Life at High Altitudes
PROFESSOR SIR B. H. C. MATTHEWS, C.B.E., M.A., SC.D., F.R.S. *(Cambridge)*

*The Physiological Effects of Gravity
GROUP CAPTAIN W. K. STEWART, C.B.E., A.F.C., R.A.F. *(Farnborough)*

The Kinetics of Rapid Chemical and Biological Reactions
PROFESSOR F. J. W. ROUGHTON, M.A., PH.D., F.R.S. *(Cambridge)*

Metal Catalysis and Intracellular Respiration
PROFESSOR D. KEILIN, F.R.S. *(Cambridge)*

Biophysical Studies of Connective Tissue
PROFESSOR J. T. RANDALL, D.SC., F.R.S. *(London, King's College)*

*The Functional Significance of Connective Tissue
A. H. T. ROBB-SMITH, M.A., M.D., F.R.C.P. *(Oxford)*

*Silicosis
PROFESSOR E. J. KING, PH.D., D.SC. *(Postgraduate Medical School of London)*

Pulmonary Asbestosis
PROFESSOR MATTHEW STEWART, C.B.E., F.R.C.P. *(Leeds)*

Organ Culture in Biological and Medical Research
HONOR B. FELL, F.R.S. *(Cambridge)*

The Biology of the Foetal Membranes and the Placenta
PROFESSOR E. C. AMOROSO, M.B., B.CH., PH.D.
(London, Royal Veterinary College and Hospital)

*The Physiological Actions of the Sex Hormones
P. M. F. BISHOP, B.CH., D.M.
(London, Institute of Obstetrics and Gynaecology)

*Acid and Alkaline Phosphatase in Disease
PROFESSOR E. J. KING, M.A., D.SC., PH.D., F.R.I.C.
(Postgraduate Medical School of London)

Principles of Chromatography
R. L. M. SYNGE, F.R.S. *(Aberdeen)*

Some Recent Ideas about the Proteins and other Biological Macromolecules
PROFESSOR W. T. ASTBURY, M.A., SC.D., F.R.S. *(Leeds)*

*Stress and Thyroid Activity
PROFESSOR G. W. HARRIS, SC.D., M.D., F.R.S.
(London, Institute of Psychiatry)

The Vascular Lesions of Pituitary Necrosis
PROFESSOR H. L. SHEEHAN, D.SC., M.D., F.R.C.P., F.R.C.O.G.
(Liverpool)

*Science and History
H. E. SIGERIST, M.D., LL.B., D.LITT. *(Yale, U.S.A.)*

*Anticholinesterases
W. D. M. PATON, M.A., B.M., B.CH.
(London, University College Hospital Medical School)

*The Scientific Approach to Dermatology
R. M. B. MACKENNA, M.A., M.D., F.R.C.P.
(London, Institute of Dermatology)

*Hemispherectomy and the Localization of Function
E. A. CARMICHAEL, C.B.E., F.R.C.P.
(London, Institute of Neurology)

*Tissue Repair
PROFESSOR G. R. CAMERON, D.SC., F.R.C.P., F.R.S.
(London, University College Hospital Medical School)

The Design of Clinical Experiments
D. D. REID, M.D., PH.D.
(London School of Hygiene and Tropical Medicine)

*The Physiology of the Autonomic Nervous System
W. S. Feldberg, M.D., F.R.S.
(*National Institute for Medical Research, Mill Hill*)

*The Principles of Ganglionic Block
W. D. M. Paton, M.A., B.M., B.CH.
(*London, University College Hospital Medical School*)

*Cholinesterases and Anti-Cholinesterases
Professor R. H. S. Thompson, D M.
(*London, Guy's Hospital Medical School*)

Certain Aspects of Nerve Repair
H. J. Seddon, M.A , D.M., F.R.C.S. (*London, Institute of Orthopaedics*)

Development of the Heart in Relation to Congenital Heart Disease
Professor J. D. Boyd, M A , M.SC., M.D. (*Cambridge*)

Dynamics of the Heart Beat
Professor A. Hemingway, M.SC., M.B , CH.B. (*Leeds*)

The Natural History of Essential Hypertension
Professor G. W. Pickering, M.A., M.B., F.R.C.P.
(*London, St. Mary's Hospital Medical School*)

Causes of Hypotension in Man
Professor E P. Sharpey-Schafer, M.B., B.CHIR., F.R.C P.
(*London, St. Thomas's Hospital Medical School*)

1953–54

Scientific Method in Medical Research
Sir Henry Dale, O M , G.B.E., F.R C.P , F.R C.

*Experimental Psychopathology
Professor R W. Russell, M A , PH D., D SC.
(*London, University College*)

*Biological Synthesis
G Popják, M.D.
(*National Institute for Medical Research, Mill Hill*)

*Acetylcholine and the Maintenance of the Cardiac Rhythm
Professor J. H. Burn, M A., F R.S. (*Oxford*)

*The Growth Hormone of the Anterior Pituitary Gland
Professor F. G. Young, M.A., D.SC., F R.I C , F R.S. (*Cambridge*)

1954-55

*Why Biophysics?
PROFESSOR A. V. HILL, C.H., SC D., F.R.S.
(London, University College)

*Protein Malnutrition
PROFESSOR B. S PLATT, C.M.G , M.B., CH.B., PH D.
(London School of Hygiene and Tropical Medicine)

The Importance of Detail in the Clinical Anatomy of the Autonomic Nervous System
PROFESSOR J. D. BOYD, M.A , M SC., M D *(Cambridge)*

*The Effects of Visceral Distension
PROFESSOR D. WHITTERIDGE, D.M., F R.S. *(Edinburgh)*

Some Features of the Peripheral Circulation and Vascular Bed
P. M. DANIEL, M A , M.B , B CHIR. *(Oxford)*

*The Chemical Basis of Blood Group Specificity in Man
PROFESSOR W T. J MORGAN, D SC., PH D , F.R S
(London, Lister Institute of Preventive Medicine)

*The Electrolyte and Metabolic Response to Trauma
PROFESSOR G. M. WILSON, M.D , F.R.C P.ED. *(Sheffield)*

*Enzymes in Injury
J. D. JUDAH, B M., B.CH., M.R C.P.
(London, University College Hospital Medical School)

*Shock from Burns
J. P. BULL, M.A., M.D. *(Birmingham)*

*Histochemistry and its Application to the Basic Sciences
A. G. EVERSON PEARSE, M.A., M.D , D C P.
(Postgraduate Medical School of London)

*Comparative Anatomy of the Larynx
SIR VICTOR E. NEGUS, D.SC., M S., F R C S
(London, Ferens Institute)

Factors Influencing Adrenocortical Activity in Health and Disease
R I. S. BAYLISS, M A., M.D., M.R.C.P.
(Postgraduate Medical School of London)

*Release and Action of Posterior Pituitary Hormones
L. MARY PICKFORD, D SC *(Edinburgh)*

*The Genetics of Some Biochemical Abnormalities
PROFESSOR J. B. S. HALDANE, M.A., D.SC., F.R.S.
(London, University College)

*The Chemotherapy of Cancer
PROFESSOR E. BOYLAND, PH.D., D.SC.
(London, Institute of Cancer Research)

*Causes of Failure in Antibiotic Therapy
PROFESSOR L. P. GARROD, M.D., F.R.C.P.
(London, St. Bartholomew's Hospital Medical College)

*Reactions to Bacterial Invasion
PROFESSOR A. A. MILES, C.B.E., M.D., F.R.C.P.
(London, Lister Institute of Preventive Medicine)

*The Action of Bacterial Enzymes on Immunizing Antigens
PROFESSOR LORD STAMP, M.A., M.B., CH.B.
(Postgraduate Medical School of London)

*Antiviral Immunity
PROFESSOR H. B. MAITLAND, M.SC., M.D. *(Manchester)*

*Antimalarial Drugs
C. C. CHESTERMAN, O.B.E., M.D., F.R.C.P.
(London, Middlesex Hospital Medical School)

Chemotherapy of Tuberculosis
P. M. D'ARCY HART, M.A., M.D., F.R.C.P.

Blood Groups
R. R. RACE, PH.D., M.R.C.S., F.R.S.
(London, Lister Institute of Preventive Medicine)

Congenital Haemolytic Anaemia
J. V. DACIE, M.D., M.R.C.P.
(Postgraduate Medical School of London)

*The Supporting System and its Disorders
PROFESSOR J. H. KELLGREN, M.B., B.S., F.R.C.S., F.R.C.P.
(Manchester)

*Regulation of Body Water
PROFESSOR G. M. BULL, M.D., M.R.C.P. *(Belfast)*

1954-55

*Why Biophysics?
PROFESSOR A. V. HILL, C.H., SC.D., F.R.S.
(London, University College)

*Protein Malnutrition
PROFESSOR B. S. PLATT, C.M.G., M.B., CH.B., PH.D.
(London School of Hygiene and Tropical Medicine)

The Importance of Detail in the Clinical Anatomy of the Autonomic Nervous System
PROFESSOR J. D. BOYD, M.A., M.SC., M.D. *(Cambridge)*

*The Effects of Visceral Distension
PROFESSOR D. WHITTERIDGE, D.M., F.R.S. *(Edinburgh)*

Some Features of the Peripheral Circulation and Vascular Bed
P. M. DANIEL, M.A., M.B., B.CHIR. *(Oxford)*

*The Chemical Basis of Blood Group Specificity in Man
PROFESSOR W. T. J. MORGAN, D.SC., PH.D., F.R.S.
(London, Lister Institute of Preventive Medicine)

*The Electrolyte and Metabolic Response to Trauma
PROFESSOR G. M. WILSON, M.D., F.R.C.P.ED. *(Sheffield)*

*Enzymes in Injury
J. D. JUDAH, B.M., B.CH., M.R.C.P.
(London, University College Hospital Medical School)

*Shock from Burns
J. P. BULL, M.A., M.D. *(Birmingham)*

*Histochemistry and its Application to the Basic Sciences
A. G. EVERSON PEARSE, M.A., M.D., D.C.P.
(Postgraduate Medical School of London)

*Comparative Anatomy of the Larynx
SIR VICTOR E. NEGUS, D.SC., M.S., F.R.C.S.
(London, Ferens Institute)

Factors Influencing Adrenocortical Activity in Health and Disease
R. I. S. BAYLISS, M.A., M.D., M.R.C.P.
(Postgraduate Medical School of London)

*Release and Action of Posterior Pituitary Hormones
L. MARY PICKFORD, D.SC. *(Edinburgh)*

Thyroid Hormones
ROSALIND PITT-RIVERS, PH.D., F.R.S.
(National Institute for Medical Research, Mill Hill)

*Some Chemical Aspects of Abnormal Growth
PROFESSOR F. BERGEL, D.PHIL.NAT., D.SC., F.R.I.C.
(London, Institute of Cancer Research)

Theory and Application of the Nitrogen Mustards
PROFESSOR A. HADDOW, M.D., D SC., PH D.
(London, Institute of Cancer Research)

Medical Significance of Biochemical Lesions
PROFESSOR SIR RUDOLPH PETERS, M.A., M.D., D.SC., F.R.C.P., F.R.S.
(Oxford)

*Substances that Increase Tissue Permeability and their Relation to Infection and Fertilization
DOUGLAS MCCLEAN, M.B.,B.S.
(Lister Institute of Preventive Medicine, Elstree)

*Cooling of the Whole Organism
D. G. MELROSE, B.M., B.CH *(Postgraduate Medical School of London)*

*Radiation Effects on Bone
JANET VAUGHAN, O.B.E., D.M., F.R.C P. *(Oxford)*

Actions of Antibiotics on Bacteria
E. F. GALE, SC.D., F.R.C. *(Cambridge)*

Tissue Transplantation Immunity
PROFESSOR P. B. MEDAWAR, D.SC., F.R.S.
(London, University College)

*Correlation between Laboratory and Clinical Findings in Hypersensitivity
PROFESSOR J. R. SQUIRE, M.D., F R.C.P. *(Birmingham)*

*The Chemistry of the Porphyrias
PROFESSOR C H. GRAY, D.SC., M.D., M R.C.P., F.R.I.C.
(London, King's College Hospital Medical School)

Protein Biosynthesis
T. S. WORK, D.SC., PH D.
(National Institute for Medical Research, Mill Hill)

*Nucleoproteins in Cell Structure
PROFESSOR J. N. DAVIDSON, M.D., D SC *(Glasgow)*

*The Effects of Alcohol
PROFESSOR J. H. GADDUM, SC.D., F.R.S. (*Edinburgh*)

*Fat Metabolism
PROFESSOR A. C. FRAZER, D.SC., PH.D., M.D., F.R.C.P. (*Birmingham*)

*The Plasma Proteins
R. A. KEKWICK, D.SC.
(*London, Lister Institute of Preventive Medicine*)

*The New Look in Virus Research
C. H. ANDREWES, M.D., F.R.C.P., F.R.S.
(*National Institute for Medical Research, Mill Hill*)